BIOCHEMICAL ANALYSIS OF PROTEINS IN THE TELOMERE MAINTENANCE PATHWAY

BY

DIANE C. DEZWAAN

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Cell and Developmental Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Doctoral Committee:

Professor Andrew Belmont, Chair
Alumni Professor Martha Gillette
Associate Professor Ann Nardulli
Associate Professor Stephanie Ceman

ABSTRACT

Eukaryotic linear chromosomes culminate in nucleoprotein structures designated telomeres. The terminal telomeric DNA consists of tandem repeats of a G-rich motif that is established and maintained by the action of the specialized reverse transcriptase called telomerase. In addition to the function of telomerase, the telomere environment requires an efficient means to assemble and disassemble a multitude of structures to operate correctly and to help achieve cellular homeostasis. Distinct protein assemblies are nucleated at telomeric DNA to both guard the ends from damage and lengthen the DNA after replication. In yeast, Cdc13 recruits either Stn1-Ten1 to form a protective cap or the telomerase holoenzyme to extend the DNA. I have established an in vitro yeast telomere system in which Stn1-Ten1-unextendable or telomerase-extendable states can be observed. Notably, the yeast Hsp90 chaperone Hsp82 mediates the switch between the telomere capping and extending structures by modulating the DNA binding activity of Cdc13. The telomere length and telomerase telomere occupancy also appear to be yeast Hsp90 dependent. Taken together, my data show that the Hsp82 chaperone facilitates telomere DNA maintenance by promoting transitions between two operative complexes and by reducing the potential for binding events that would otherwise block the assembly of downstream structures.

The first telomerase cofactor identified was the budding yeast protein Est1, which is conserved through humans. While it is evident that Est1 is required for telomere DNA maintenance, understanding its mechanistic contributions to telomerase regulation has been limited. In vitro, the primary effect of Est1 is to activate telomerase-mediated DNA extension. Although Est1 displayed specific DNA and RNA binding, neither activity

contributed significantly to telomerase stimulation. Rather Est1 mediated telomerase upregulation through direct contacts with the reverse transcriptase subunit. My studies provide insights into the molecular events used to control the enzymatic activity of the telomerase holoenzyme.

DEDICATIONS

I would like to dedicate my thesis to the four people who had a significant influence on my life's trajectory during my graduate school career. First to Tunji Toogun, you have been in my thoughts and prayers every SINGLE day since the moment we lost you. You lit up a room with your wonderful smile and you taught me what the definition of hard work really meant. Thank you so very much for being my friend, I miss you and will always remember the time we shared with a smile.

To my father, who has been a constant pillar of support throughout my entire life. You taught me how to be strong in the face of adversity and imparted the best and most useful advice to me, "As long as you keep on the sunny-side of life, you can overcome any obstacle". I love you so much and am lucky to have such a genuinely good person as a Dad.

To Rob, what more can I say than I simply love you with all my heart and hope to show you that everyday of my life.

And finally to my professor, Dr. Brain Freeman, who knew that "low hanging fruit" would be so hard to pick, but in the end we got it! I never realized when I started graduate school that not only would I be privileged to have such a knowledgeable and influential mentor scientifically, but also that I would gain a friend who has conveyed so much wisdom and so many life lessons to me. Even with all my quirks, you never gave up on me and continued to push me to strive for excellence. After all of these years, the backbone that you had on back-order at Fischer may have finally arrived. Thank you Brian, I am eternally grateful.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION……..….1

CHAPTER 2: HSP90 AND THE “CST” CAPPING COMPLEX AT THE TELOMERE.....18

CHAPTER 3: BIOCHEMICAL CHARACTERIZATION OF EST1……...…40

CHAPTER 4: DISCUSSION…….…..62

CHAPTER 5: MATERIALS AND METHODS……..…...73

REFERENCES……110

APPENDIX A: BIOCHEMICAL CHARACTERIZATION OF FULL LENGTH CDC13 PURIFIED FROM SmP *E. coli* ……...…….119

APPENDIX B: BIOCHEMICAL CHARACTERIZATION OF EST3…….126

AUTHOR’S BIOGRAPHY……..….131

CHAPTER 1*

INTRODUCTION

The cell interior is a highly dynamic system. In live cells most proteins move with apparent diffusion coefficients (0.2–20 μm2s-1) yet within this ostensible chaos the separate parts routinely and efficiently organize into multi-step pathways to transduce signals or accomplish requisite work (Mitchison, 1992; Misteli, 2001; Bubulya and Spector, 2004; Trinkle and Lamond, 2007). The rapid kinetics displayed by individual proteins in vivo was not expected since isolated biological complexes are often inherently stable in vitro and it had been predicted that the crowded cell interior would impede movement (Ellis, 2001; Kushner, 1969; Perham, 1975). However, using advanced imaging techniques it is becoming apparent that most cellular components move rapidly (Bubulya and Spector, 2004; Lippincott-Schwartz *et al*., 2000). Despite the visual evidence for such fast movements, it is often unclear which factors and events convert seemingly stoic in vitro structures into dynamic and efficient systems in vivo.

The physiological need for pathways to work both rapidly and selectively within the crowded milieu of the cell interior presents great challenges for achieving homeostasis in the midst of fluctuating internal and external stimuli. Early biochemical work with isolated proteins demonstrated that the specificity of biological complex formation can be attributable to cooperative interactions between the individual subunits

* Figures in this chapter were modified from the following publications 1.) **DeZwaan DC** and Freeman BC. (2010) HSP90 manages the ends. *Trends Biochem. Sci.*, 35: 384-91. 2.) **DeZwaan DC** and Freeman BC. (2010) Is there a telomere-bound 'EST' telomerase holoenzyme? *Cell Cycle*, 9:1913-17.

(Garel *et al.*, 1984). While cooperative associations provide a mechanism for rapid assembly, the inherent stability of such organized structures would likely interfere with the timing necessary for biological systems. Further complicating proper function is the nature of the cell interior: it is densely packed (e.g., the intracellular macromolecular concentration is ~350–400 mg ml-1) and often contains multiple binding partners for each protein—both features increase the probability for non-productive or off-pathway interactions (Minton, 2006). Though it would be possible to avoid these problems if proteins assembled into "holo" structures that contain all the components necessary to achieve function, this solution is not practical given the crosstalk between and variety of cellular paths.

Recent proteomic studies underscore the limited use of stable protein structures in vivo, as there appear to be only ~500–800 "core" protein complexes that average 3–5 subunits (Gavin *et al.*, 2002; Krogan *et al.*, 2006). To achieve the diversity necessary for life, either individual proteins or the core structures form metastable interactions with a variety of accessory factors. The fully assembled complexes would define discrete steps along a given pathway and would be dependent upon a dynamic organization in order to insure an effective progression. To build each structure with the correct subunits (i.e., avoid inappropriate partners) and to foster efficient transitions between the functional complexes, cellular factors likely have evolved to destabilize each protein assembly, which would be imperative for multi-step pathways. While it is possible that the steady-state stabilities of individual structures are mediated by unique coevolved factors, the probability of this scheme is low, as it would require thousands of additional proteins.

Instead, I suggest that molecular chaperones serve to promote a general and continuous dynamic protein environment within the cell.

To illustrate the potential complexities and obstacles faced by most, if not all, biological pathways, I focused on the impact of one molecular chaperone network on a single cellular pathway. Specifically, I investigated the potential points of influence that the heat shock protein 90 (HSP90) chaperone machinery has on telomere protein biology. By coordinating the assembly and disassembly of numerous telomere complexes, I speculate that HSP90 and its associated co-chaperones facilitate a precise and efficient working environment that is highly beneficial for telomere function. Once a better understanding of Hsp90's role at the telomere had been established, I further investigate components directly involved in the dynamic nature of the telomere maintenance pathway. Thus, I focus on the characterization of an integral telomerase protein component called Est1, whose dynamics appear to play a central role in perpetuating a functional telomere extension cycle.

The Telomere Protein System

Telomeres consist of a tract of G-rich DNA motifs culminating in a 3' single-stranded overhang (telomeric DNA) that is recognized by a collection of associated factors (telomere-binding proteins) that maintain the terminal DNA at an appropriate length to preserve genome stability and cell viability (Gilson and Geli, 2007). A multitude of events must occur at telomeres to achieve function and all of the activities are challenged by a need to work precisely and efficiently within a limited time-frame. Notably, a single shortened telomeric end is sufficient to induce cellular senescence,

whereas an unregulated telomere system can lead to malignant growth (Shay and Wright, 1996; Hemann *et al.*, 2001). Hence, telomere biology presents an ideal molecular model to further our understanding of the cellular mechanisms that are required to proficiently direct and drive a pathway, which affects homeostasis.

Multiple protein complexes assemble at telomeres to execute specific functions that are required for protection and extension of the terminal DNA (Gilson and Geli, 2007; Shore and Bianchi, 2009). In general, the complexity of the telomere varies in different stages of the cell cycle; however, specific cellular needs can also direct molecular decisions. For instance, telomeric DNA is extended only in S-phase, but not all telomeres are extended in every cell cycle (Marcand *et al.*, 2000). In a short temporal window (<15 min) during late S-phase, a decision is made on the basis of the length of the double-stranded telomeric tract (i.e. the number of telomeric repeats are essentially counted) to either generate a telomerase-extendable state or form an unextendable structure (Teixeira *et al.*, 2004).

If a telomeric end is approaching a critically shortened state, which would trigger cellular senescence if reduced further, then the 3' overhang will be extended (Hemann *et al.*, 2001). Extension of the DNA requires several different aspects of S-phase-specific activity, including DNA resectioning, extending and fill-in replicating, which are all mediated by distinct protein complexes (Figure 1). Interestingly, components of each assemblage are capable of binding to the single-stranded, G-rich overhang, which presents the opportunity for inhibitory competitive DNA interactions. Yet, all three events operate efficiently within the allotted narrow time window following DNA replication in S phase. If, however, the telomeric DNA tract is sufficiently long, the

structure will remain in an unextendable or capped state, although the composition of the cap will vary as the cell cycle progresses (Figure 1) (Gilson and Geli, 2007; Shore and Bianchi, 2009). Hence, the telomere environment must be maintained in a dynamic state in order to respond to the signals that dictate function and to perform the work that is required to maintain the chromosomal termini. But what mechanism(s) are used to properly assemble (i.e. avoid competitive binding) and disassemble (i.e. transition between structures) the various telomere complexes?

Eukaryotic Molecular Chaperone Network

Molecular chaperones comprise a diverse protein family that is defined primarily by an in vitro activity (i.e. ability to suppress non-native protein aggregation) rather than by a conserved amino acid sequence or domain (Ellis and van der Vies, 1991). Chaperone family members generally display a promiscuous protein-binding capacity with an affinity for short hydrophobic amino acid motifs that likely accounts for the shared ability to suppress non-native protein aggregation in vitro (Hendrick and Hartl, 1993). Chaperones typically have short-lived, low affinity interactions with a client, which offsets the broad binding capacities and avoids interfering with the activity of a target protein. These two evolved characteristics make molecular chaperones ideal candidates to facilitate a functional dynamic environment comprised of a wide array of proteins.

The eukaryotic molecular chaperone system is coordinated primarily around the two highly abundant chaperones HSP90 and HSP70 along with their cognate cochaperones (Wegele *et al*., 2004; Mayer and Bukau, 2005). Although many types of cellular activity have been identified for the HSP70 network, the general roles of HSP90

and its many co-chaperones in vivo (e.g. p23, CDC37, AHA1, and SGT1) remain elusive, despite their conservation within eukaryotes. The HSP90 chaperone complex was identified originally in stable association with signaling proteins (i.e. kinases and steroid receptors) and it has been argued that this chaperone network serves to maintain metastable factors in forms that can be readily activated (Pratt and Toft, 2003). However, recent proteomic and genetic interaction studies suggest that signaling protein maintenance might be the tip of the iceberg for the duties of the eukaryotic HSP90 chaperone machinery (Millson *et al.*, 2005; Zhao *et al*., 2005; McClellan *et al*., 2007).

Based on evolutionary considerations, cellular reliance on the HSP90 chaperone system has increased significantly. Prokaryotes typically contain a single non-essential HSP90 gene (e.g. HtpG in Escherichia coli), yet eukaryotes often have multiple HSP90 genes that are essential and have acquired a collection of co-chaperone partner proteins that do not appear in prokaryotes (Johnson and Brown, 2009). By contrast, HSP70 proteins are widely regarded as one of the best conserved proteins across the three domains of life (Mayer and Bukau, 2005). Yet the functional activities of HSP70 have apparently changed as involvement with DNA-associated events has lessened (Ziemieowicz *et al.*, 2001). Although the workload shift in the chaperone system likely has numerous important physiological consequences, I suspect one key change has been employment of the HSP90 machine to foster homeostasis by modulating protein–DNA dynamics.

Historically, molecular chaperones have been recognized as factors that mediate the association and disassociation of protein complexes (Ellis, 2007). Recent studies indicate that certain chaperones serve comparable roles with protein–DNA structures

(Shaknovich *et al.*, 1992; Freeman and Yamamoto, 2002; Muller *et al.*, 2004; Stavreva *et al.*, 2004; Walerych *et al.*, 2004; Toogun *et al.*, 2007; Toogun *et al.*, 2008; DeZwaan *et al.*, 2009). For instance, several molecular chaperones, including HSP90, HSP70 and p23, can promote protein–DNA dynamics of diverse targets, including components involved in RNA transcription, telomere maintenance, DNA repair and DNA replication (DeZwaan and Freeman, 2008; Konieczny and Zylicz, 1999; Richter *et al.*, 2007; Hager *et al.*, 2009). One aim of my thesis was to focus on the role of the HSP90 chaperone system in modulating the assembly and disassembly of telomere structures.

The Hsp90 Machine Associates with the Telomerase Enzyme

The initial connection between HSP90 and telomere components was made while attempting to identify cellular factors required for telomerase holoenzyme function (Holt *et al.*, 1999). The authors discovered that HSP90 and the p23 co-chaperone interact with the protein subunit of human telomerase (hTERT) through a yeast two-hybrid screen. Telomerase is a specialized reverse transcriptase (RT) in which the core enzyme comprises a protein module (TERT in humans and Est2 in yeast) and an RNA subunit (human TR and yeast TLC1) (Gilson and Geli, 2007; Shore and Bianchi, 2009). Telomerase utilizes the RNA as a template to extend the 3' chromosomal ends by 100 nucleotides or more per cell cycle by reiteratively appending a short (6–8 bases) telomeric DNA repeat during each binding event (Teixeira *et al.*, 2004; Britt-Compton *et al.*, 2009). Core human telomerase is sufficient for robust DNA extension activity in vitro but core yeast telomerase is not (Cohn and Blackburn, 1995; Morin, 1989). Nonetheless, both homologs rely on a multitude of additional proteins for proper control; HSP90 and

p23 represent the first two proteins shown to interact directly with telomerase and contribute to its enzymatic activity (Holt *et al*., 1999).

The link between telomerase and molecular chaperones was extended by the finding that additional chaperones, including HSP70, HSP40 and HOP (HSP90/HSP70 organizing protein), associate with the core human enzyme, presumably to assemble the RT and RNA template in vitro (Forsythe *et al*., 2001). This observation fits with the classic steroid receptor–chaperone model in which chaperones are required to generate and maintain the hormone-binding state of receptors through a stable interaction that persists until the receptor is hormone-activated (Pratt and Toft, 2003). Surprisingly, HSP90 and p23 remain associated with telomerase even after extending a telomeric DNA substrate, suggesting that these chaperones have a role beyond telomerase protein folding (Forsythe *et al*., 2001). Notably, both HSP90 and p23 also function with DNA-associated steroid receptors to promote a dynamic DNA-binding cycle that is required for proper gene regulation (Freeman and Yamamoto, 2002; Stavreva *et al*., 2004). If HSP90 and p23 regulate both telomerase and receptors at or near DNA, then perhaps these chaperones have a general cellular function with diverse DNA-binding proteins.

Unpredictably, an early yeast genetic study showed that over-expression of either Hsp82 or Hsc82 (the two yeast HSP90 isoforms) led to telomeric DNA shortening, thus suggesting a negative effect of Hsp82/Hsc82 in telomere DNA maintenance in vivo (Grandin and Charbonneau, 2001). The cause of the decreased telomere DNA length was not apparent; however, the potential for Hsp82 to affect telomere-binding proteins besides telomerase was revealed when HSP82 was identified as a high-copy suppressor of two mutations (cdc13-1 and stn1-157) known to alter telomere DNA length (Grandin

and Charbonneau, 2001). Hence, HSP90 proteins might affect multiple telomere associated proteins.

The observation that telomeric DNA length was essentially normal in a strain expressing limited Hsp82 levels (10% of normal) further supported the contention that Hsp82 does not have a positive effect on telomerase (Grandin and Charbonneau, 2001). An Hsp82 protein reduction tactic was used because the eukaryotic HSP90 genes are essential and therefore it is not possible to test for in vivo effects in the absence of an HSP90 protein. It should be noted, however, that normal yeast has 500,000 Hsp82 molecules per cell (Ghaemmaghami *et al*., 2003). Thus, if telomerase is a high-affinity substrate for Hsp82, a change in telomeric DNA length might not be expected in yeast expressing 50,000 Hsp82 molecules.

At the very least, the two initial reports investigating the connection between HSP90 and telomere proteins suggested that the relationship is complicated. Although the discord between the in vitro and in vivo data might be unsatisfying, it is not entirely surprising given the nature of the cellular chaperone network (i.e. abundant and promiscuous proteins). For example, HSP70 is the fundamental nascent chain-binding protein in vivo (Beckmann *et al*., 1990). Yet, loss of the E. coli HSP70 gene DnaK results in only mild phenotypes (Bukau and Walker, 1989). However, in the absence of both DnaK and the trigger factor (TF) molecular chaperone, cells die owing to massive protein aggregation (Deuerling *et al*., 1999). Nonetheless, based on in vitro assays, a role for DnaK in protein folding had been long accepted before the discovery of the synthetic lethal phenotype between DnaK and TF. Therefore, in vitro studies can identify important chaperone functions that are not readily apparent by typical in vivo work given the

redundant and/or compensatory nature of the cellular molecular chaperone system. To dissect the functional role of HSP90 with telomerase, suitable genetic conditions, in conjunction with a more detailed biochemical analysis, were necessary.

Combating the End Replication Problem

A lack of functional knowledge also surrounded many of the key telomerase co-factors, whose dynamics had been speculated to assist in avoidance of the "end replication problem" associated with telomeric ends. The "end replication problem" is a dilemma that challenges all linear chromosomes (Watson, 1972; Olovnikov, 1973). The DNA replication machinery relies on an RNA primer to initiate synthesis and removal of the extreme 5' RNA oligonucleotide leaves a gap that cannot be filled in by the conventional cellular DNA polymerases (Blackburn, 1991). Thus, the lagging strand would shorten with each cell division in the absence of a compensatory mechanism. In addition, the leading strand can be trimmed by 5'-exonuclease activities that are coupled to passage of the replication fork (Gilson and Geli, 2007). Several plausible routes were proposed to correct these limitations including the involvement of a nucleotide terminal transferase, recombinase or a novel enzyme that might elongate the 3' ends (Watson, 1972; Olovnikov, 1973; Shampay *et al.*, 1984). Ultimately, it was found that a specialized reverse transcriptase termed telomerase, which is comprised of protein enzyme and RNA template, is required to maintain linear chromosomes by elongating the 3' terminus (Greider and Blackburn, 1987; Blackburn *et al.*, 1989).

To investigate how chromosomal termini are maintained a genetic screen in budding yeast was employed that exploited a cellular senescence phenotype that occurs

upon telomere dysfunction (Lundblad and Szostak, 1989). Telomeric DNA length was measured in the genetic isolates, as a secondary screen. This approach revealed the first telomere-associated factor *EST1* (Ever Shorter Telomere 1) since the chromosomal terminal DNA in *est1*Δ yeast is progressively lost and the cells coordinately reach replicative senescence. In a follow-up screen three additional components (*EST2*, *EST3* and *EST4*) were uncovered (Lendvay *et al.*, 1996). Further analysis identified the *EST4* gene to be synonymous with *CDC13*, which was previously found in a temperature sensitive screen used to isolate Cell Division Cycle (CDC) mutants (Hartwell *et al.*, 1973). Eventually, it would be realized that these seminal studies discovered the gene encoding the telomerase reverse transcriptase Est2, two telomerase regulatory proteins (Est1 and 3) and a vital telomeric DNA-binding protein (Cdc13) required for both extending and protecting telomeres (Taggart and Zakian, 2003). Importantly, the four EST proteins appear to have structure/function homologs in humans (Linger and Price, 2009). A separate study described an RNA component Tlc1 that forms the core telomerase enzyme in conjunction with Est2 (Singer and Gottschling, 1994). Together, these five factors contribute to a critical cellular machine that is necessary to lengthen telomeric DNA in budding yeast. However, despite intensive efforts, the precise functional contribution made by each factor to telomere maintenance still has not been entirely delineated.

An early model proposed a holoenzyme complex formed by the *EST1-4* and *TLC1* gene-products, which was supported by an epistasis analysis showing no apparent enhanced telomere or senescence phenotypes upon combinatorial deletion of the various loci (Lendvay *et al.*, 1996). Biochemical studies in which telomerase activity was

isolated in an enriched extract along with additional genetic work indicated that Est2 and Tlc1 form the core telomerase enzyme with Est2 serving as a reverse transcriptase protein and Tlc1 as an RNA template (Singer and Gottschling, 1994; Counter *et al*., 1997; Lingner *et al*., 1997). Coimmunoprecipitation experiments suggested that Est1 initiates formation of a minimal holoenzyme complex by directly binding to the Tlc1 RNA (Steiner *et al*., 1996; Zhou *et al*., 2000). Further studies suggested an assembly pathway in which Est1 and Est2 bind directly to the Tlc1 RNA and Est3 joins the complex in an Est1-dependent manner and associates with the amino-terminus of Est2 (Hughes *et al*., 2000; Livengood *et al*., 2002; Seto *et al*., 2002; Friedman *et al*., 2003; Osterhage *et al*., 2006). In the absence of Tlc1, stable biochemical interactions between Est1 and Est2 were not apparent, although contact between Est1 and Est2 in the absence of Tlc1 can be detected using the chromatin immunoprecipitation assay in vivo (Evans and Lundblad, 2002; Bianchi *et al*., 2004). As free core telomerase (*i.e*., non-telomere bound) does not form a "precipitable" interaction with Cdc13, Cdc13 has not been considered a holoenzyme component (Hughes *et al*., 2000). Together, these studies indicated that Tlc1 and the Est1-3 proteins form a nominal holoenzyme complex that stably exists independent of a DNA-bound telomere environment.

The Role of Est1 and Cdc13 in Telomerase Function

Est1 and Cdc13 each have an in vitro ability to bind single-stranded G-rich DNA, which is representative of the chromosomal 3' telomeric DNA overhangs (Nugent *et al*., 1996; Vitra-Pearlman *et al*., 1996; Qi and Zakian, 2000). Hence, it was suggested that Est1 and Cdc13 mediate telomerase recruitment to a telomere. Supporting this notion was the capacity of Est1 and Cdc13 to interact directly, as shown by yeast two-hybrid and

coimmunoprecipitation assays (Nugent *et al.*, 1996; Qi and Zakian, 2000). Genetic studies solidified a now classic telomere model and introduced the concept that Est1 might function as a bridging protein connecting telomerase to DNA-bound Cdc13 (Figure 2) (Hughes *et al*., 2000; Nugent *et al*., 1996; Evans and Lundblad, 1999; Pennock *et al*., 2001). Perhaps, the most prominent contribution to the Est1 bridging model was the observation that *est1*Δ yeast do not display an EST phenotype if the cells express a Cdc13-Est2 fusion protein (Evans and Lundblad, 1999). According to the model, Cdc13-Est2 alleviated the need for Est1 since telomerase was now covalently attached to the primary telomeric DNA binding protein Cdc13. Intriguingly, the fusion approach also demonstrated that Est1 serves a telomere role that is distinct from bridging. If Est1 was expressed in conjunction with the Cdc13-Est2 fusion then the telomeric DNA was hyperelongated, which suggests Est1 up-regulated telomerase DNA extension activity when the enzyme was in stable association with Cdc13 at a telomere.

Further genetic evidence added to the concept that an interaction between Est1 and Cdc13 was central for telomere DNA maintenance. The first data indicating a connection was the demonstration that Est1 overexpression partially suppressed the telomere shortening and cellular senescence phenotypes observed in the *cdc13-2* mutant background; (Nugent *et al*., 1996) the *cdc13-2* mutation results in a single amino acid change (E252K). Significantly, an *est1* mutation (*est1-60*) was isolated that functions as a *cdc13-2* suppressor and reestablishes near wild-type telomere maintenance and cell growth (Pennock *et al*., 2001). The Est1-60 mutant contains a single altered residue (K444E). The requirement of the 252/444 salt-bridge was confirmed with another derivative series (Cdc13-9 (E252R) and Est1-62 (K444D)) (Pennock *et al*., 2001). With

the charge-swap nature of the Cdc13 and Est1 derivatives and phenotypic connections, the requirement of a physical link between Est1 and Cdc13 for telomere DNA maintenance was well established. In general, the interaction was modeled as a bridging function even though the genetic data could also fit with roles in activation and/or stabilization. To resolve the precise contributions made by each non-core EST protein (*i.e.*, Est1, 3 and 4) would require more directed research.

The development of a telomere-specific chromatin immunoprecipitation (ChIP) protocol permitted a more mechanistic exploration of the timing and impact of select proteins on telomere complex assembly (Taggart *et al*., 2002). Est1 and Est2 were both found at telomeres in late S-phase as expected since telomeric DNA is only lengthened at this point in the cell cycle (Taggart *et al.*, 2002; Marcand *et al*., 2000). Cdc13 is apparently telomere-associated throughout the cell cycle with an increased localization during late S-phase, which likely supports its roles in a number of functions including DNA-end protection and extension (Nugent *et al*., 1996). Unexpectedly, Est2 but not Est1 localized to telomeres in G1 (Taggart *et al.*, 2002). Thus, Est1 is not requisite for telomere recruitment of telomerase. Further studies showed that yKu70/80 brings telomerase to the telomere in G1 and also influences the S-phase complex by binding to a RNA stem-loop in Tlc1 (Stellwagen *et al.*, 2003; Fisher *et al*., 2004).

Notably, the first ChIP telomere report demonstrated that both Est1 and Est2 associate normally with telomeres in a *cdc13-2* background (Taggart *et al*., 2002). This result was in stark contrast to the genetic model predicting a requisite interaction between Cdc13 and Est1, through the 252/444 salt-bridge, for recruitment of telomerase to a telomere. In follow-up work that utilized epitope variants of Est1 and Est2, reductions in

telomere interactions were detected in the *cdc13-2* background (Chan *et al.*, 2008). Though both ChIP studies employed myc-tagged version of Est1 and Est2, the second report incorporated a linker between the epitope-tag and the coding regions that presumably enhanced the sensitivity of the ChIP output (Taggart *et al*., 2002; Chan *et al.*, 2008). However, in a separate report focusing on Est1, the Est1-60 mutant displayed a telomere association that was comparable to wild-type Est1 (Smith *et al*., 2003). If Est1 requires the 252/444 salt-bridge to interact with Cdc13 and be telomere associated, then no telomere binding should have been detected for Est1-60. Collectively, the ChIP data demonstrates that the salt-bridge interaction between Est1 and Cdc13 is not requisite for nucleating holoenzyme components to a telomere. However, the salt-bridge does appear to be essential for the healing of double-stranded breaks through the recruitment of telomerase activity (Bianchi *et al.*, 2004). If the Est1 Cdc13 interaction is not central for telomerase nucleation at a telomere then why is the salt-bridge critical for telomere DNA maintenance and how might telomerase be recruited to a telomere? Thus, to better understand both the role of Hsp90 and Est1 with telomerase, an indepth biochemical analysis of each was necessary. My thesis centers around using biochemical techniques to elucidate how both Hsp90 and Est1 function within the telomere maintenance pathway.

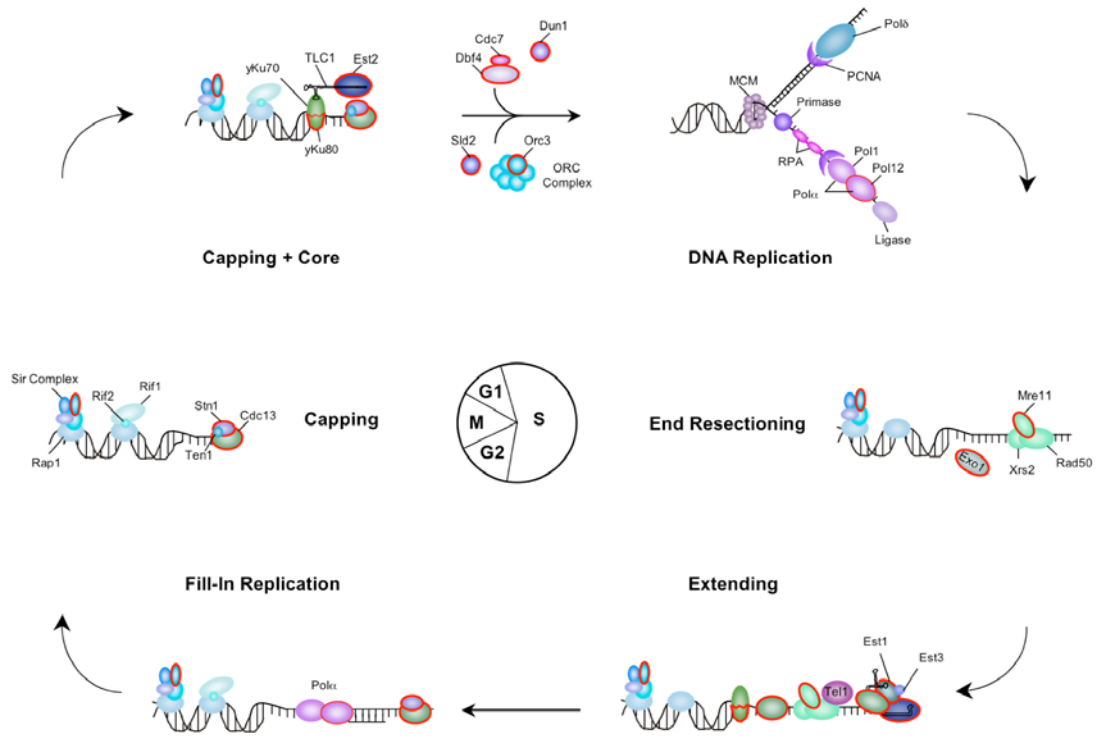

Figure 1. **The complexity of the telomere maintenance pathway** To effectively operate, the telomere system nucleates a number of different protein complexes to chromosomal ends on a cell cycle and need basis (Gilson and Geli, 2007). In M phase, a telomere is in a capped, non-extendable state to prevent DNA damage and non-homologous end joining (i). Although the telomere remains non-extendable in G1, the complex is modified by the addition of the core telomerase subunits (ii). In S phase the DNA replication machinery duplicates the DNA with the exception of the extreme 5' terminus (iii). Following replication, the DNA resectioning machinery degrades a section of the 5' C-strand (iv). In late S phase the telomere can transition to an extendable state by assembling the telomerase holoenzyme to lengthen the 3' G-strand (v). In S/G2, the C-strand is filled in by the DNA polymerase a machinery and the telomere structure returns to a protective capping complex (vi). The identities of the individual components or complexes are indicated. Interactions between HSP90

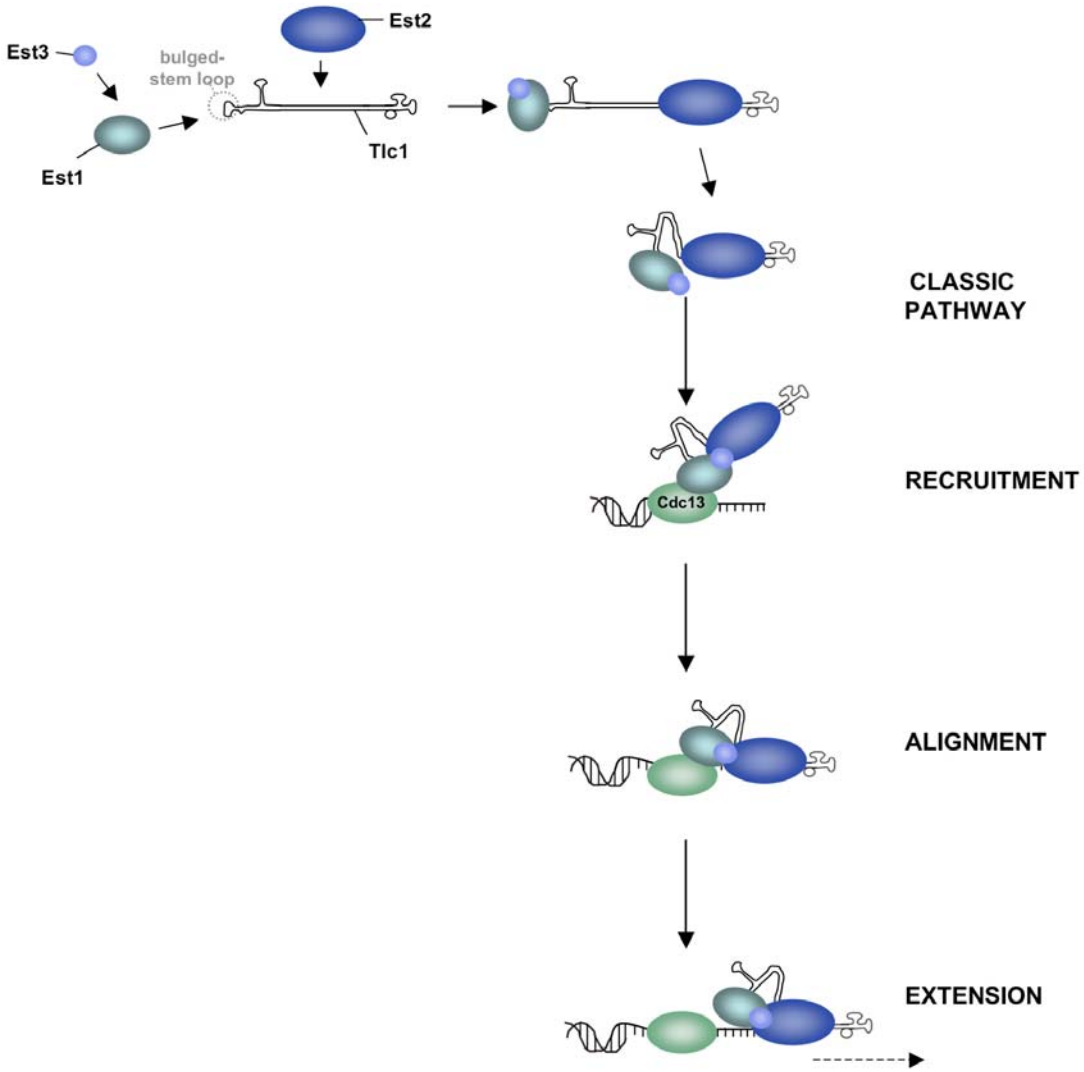

Figure 2. Genetics suggest Est1 functions as a bridging factor to recruit telomerase to the telomere. Prior studies have demonstrated that assembly of a telomerase holoenzyme initiates with Est1 and Est2 binding to the Tlc1 RNA and then the Est3 subunit enters. These steps likely occur independent of a telomere environment. Genetic analysis initially favored a "Classic" model for engaging the Est1-3 enzyme with telomeres. In the Classic recruitment pathway the Est1-3 enzyme loads onto a telomere by using the Est1 subunit as a bridging protein that connects telomerase to the telomere-bound Cdc13 protein (Nugent *et al.*, 1996; Evans and Lundblad, 1999; Hughes *et al.*, 2000). The salt-linkage between Est1 and Cdc13, which is critical for telomere DNA maintenance (Pennock *et al.*, 2001), is necessary to form the Est1/Cdc13-bridge.

CHAPTER 2*

HSP90 AND THE "CST" CAPPING COMPLEX AT THE TELOMERE

The Hsp90 molecular chaperone is a highly conserved and abundant protein that has evolved into an essential eukaryotic protein (Borkovich *et al*., 1989; Ghaemmaghami *et al*., 2003; Wegele *et al*., 2004). Given recent proteomic and genetic screens, Hsp90 has a role in a multitude of normal cellular functions and is involved in a number of diseases ranging from conformational protein folding problems to cancer (Richter *et al*., 2009). Accelerating the interest in Hsp90 is its developing use as a therapeutic target for diverse diseases (Power and Workman, 2006; Whitesell and Lindquist, 2005). Presumably, the broad therapeutic spectrum results from Hsp90's role in maintaining the conformation, stability and activity of many key cellular proteins that includes intracellular hormone receptors, cRaf, Her2, Akt, Cdk4, p53 and telomerase. Despite its apparent central role in the eukaryotic molecular chaperone system and disease relevance, its mechanistic role in client protein regulation is not well understood. In this chapter I investigate the yeast Hsp90 contributions to telomerase activities.

Telomerase maintains genomic integrity, in part, by preserving chromosome length following DNA replication (Cech, 2004; Smogorzewska and de Lange, 2004). Since conventional DNA polymerases require priming events to initiate synthesis, the

* Data presented in this chapter were originally published in 1.) Toogun OA, **DeZwaan DC** and Freeman BC. (2008) The HSP90 molecular chaperone modulates multiple telomerase activities. *Mol. Cell. Biol.*, 28:457-67. 2.) **DeZwaan DC**, Toogun OA, Echtenkamp FJ and Freeman BC. (2009) The Hsp82 molecular chaperone promotes a switch between unextendable and extendable telomere states. *Nat. Struct. Mol. Biol.*, 16:711-6. I performed all of the presented experiments in this study.

extreme terminus of each lagging strand cannot be completed—commonly referred to as the end replication problem (Watson, 1972). In the absence of a compensatory process, this limitation would lead to chromosome erosion with each round of replication. Almost all eukaryotes circumvent this problem by adding a tandem array of simple sequence repeats to each terminus that buffers against the loss. Depending upon the organism, telomerase increases chromosome ends between a few hundred to a few thousand nucleotides to create the telomeric DNA end (Smogorzewska and de Lange, 2004). Perhaps unexpectedly, it was realized that the length of each telomere is not added at once but rather telomerase typically appends 6-8 nucleotides per binding event (Prescott and Blackburn, 1997). While it had been argued that telomerase might not need to be processive to maintain telomere length (Lundblad and Szostak, 1989), recent studies in yeast indicate that telomeres can be extended over 100 nucleotides per cell cycle (Marcand *et al*., 1999; Teixeira *et al*., 2004). The mechanism(s) that govern the necessary repetitive telomerase-DNA binding and extension events are poorly understood.

The Hsp90 and p23 molecular chaperones were the first two telomerase cofactors found to alter DNA extension activity in vitro (Holt *et al.*,1999). At the time it was suggested that these two chaperones serve to assemble the telomerase catalytic protein with its RNA template—telomerase is a specialized reverse transcriptase that uses an RNA moiety to specifically extend the 3' DNA end (Smogorzewska and de Lange, 2004). However, recent reports indicate that Hsp90 and p23 can function after assembly. DNA extension by the mature telomerase enzyme is reduced upon Hsp90 inhibition (Keppler *et al.*, 2006). However, if a telomeric DNA substrate is added prior to the

Hsp90 inhibitor the effect is alleviated, which suggests Hsp90 supports telomerase DNA binding. The yeast p23 homolog Sba1p, on the other hand, was shown to promote telomerase dissociation from DNA in vitro and affect telomere maintenance and telomerase telomere occupancy in vivo (Toogun *et al*., 2007). Unfortunately, correlative in vivo roles for human Hsp90 have not been reported.

In contrast to the mammalian system, Hsp90-dependent changes to yeast telomerase activity in vitro have not been shown and effects on telomere length have been conflicting. In one report, telomere length was unaltered upon disruption of either one of the two yeast Hsp90 genes *HSP82* or *HSC82*; in addition, no telomere changes were observed in yeast expressing only low Hsp82p levels (~10% of normal) (Grandin and Charbonneau, 2001). Yet in a systematic evaluation of ~4,800 haploid gene deletion strains, the *hsc82* null was found to have shortened telomeres (Askree *et al.*, 2004). In an attempt to better understand the role(s) yeast Hsp90 proteins might have with telomerase I investigated Hsp82p-mediated effects on telomerase extension activities in vitro.

Yeast telomere length is Hsp90-dependent

To address whether proper telomere maintenance is yeast Hsp90-dependent, a southern blot analysis was necessary. While a telomere length defect might be more striking if the cellular yeast Hsp90 (*i.e.*, Hsp82p and Hsc82p) activities could be completely eliminated, the essential nature of this protein family prohibits this test (Borkovich *et al.*, 1989). As a prior study indicates that even low Hsp82p levels are sufficient to support telomere length (Grandin and Charbonneau, 2001), an alternate tactic was used in which telomere length was assessed in the absence of any wild type

Hsp82 or Hsc82 protein. To achieve this end point established engineered yeast strains were used, in which the endogenous *HSP82* and *HSC82* genes have been disrupted and viability is maintained by plasmid-born expression of either a wild type (*HSC82* or *HSP82)* or a mutant allele (Nathan and Lindquist, 1995; Johnson *et al*., 2007; Toogun *et al*., 2008). In general, both wild type Hsp82p or Hsc82p expression were sufficient for telomere DNA length maintenance, but when both wild type proteins were absent a wide telomere DNA phenotype distribution was observed in the 24 different alleles examined. The *hsp82* G170D allele was found to display the most significant decline in telomere length (~50%) (Toogun *et al*., 2008). Hence, the *hsp82/hsc82* allele-specific phenotypes indicate that a wild type yeast Hsp90 protein is required for proper telomere DNA length maintenance.

Telomerase DNA extension activity is yeast Hsp90-dependent

To address whether the observed telomere shortening might arise from a telomerase activity defect I prepared standard telomerase DEAE extracts from various allelic backgrounds (Prescott and Blackburn, 1997). In general, I found a reduced telomerase DNA extension activity in the mutant backgrounds (Figure 3A); the relative activity of each extract compared to the parental strain is provided. As the extracts all contained equivalent telomerase levels (*i.e.*, TLC1 amounts) (Figure 3A), the changes in the DNA extension activity likely result from the mutations in either Hsp82p or Hsc82p. With the exception of the S481Y and G309S backgrounds, I observed a good correlation between the relative DNA extension levels in vitro and telomere lengths in vivo (Toogun *et al.,* 2008). Given the general correlation between the in vitro and in vivo defects, I

suggest that the primary supporting role for Hsp82p and Hsc82p at the telomere is with telomerase. Importantly, titration of recombinant Hsp82p into the various extracts was sufficient to recover extension activity (Figure 3B); supplementation of a control protein (*e.g.*, BSA) had no apparent effect (data not shown). In addition to the general increase in DNA extension levels, Hsp82p supplementation promoted longer telomerase products as the pronounced pausing at the +2 position in the unsupplemented reactions was relieved (Figure 3B). These data provide the first evidence that Hsp82p might modulate nucleotide processivity by telomerase.

To more directly assess whether Hsp82p has a role in telomerase nucleotide addition, I performed DNA extension assays under limiting concentrations of nucleotide. I reasoned if Hsp82p promotes telomerase nucleotide addition then the enzyme might display an increased reliance on nucleotide availability in the absence of a wild type Hsp90 chaperone. In the assays, I exploited a telomeric DNA substrate terminating with TGG at the 3' end. Alignment of the TLC1 template and this substrate would permit incorporation of a single G nucleotide and then depending upon the availability of dTTP the complex would pause (Prescott and Blackburn, 1997). As expected I observed a single extension product at +1 in the presence of only [^{32}P]a-dGTP using extracts prepared from either wild type, G170D or T101I yeast (Figure 4, lanes marked with a minus sign). However, as dTTP is titrated into the reactions longer extensions are observed (Figure 4). While the intensity of the +1 products varied between the wild type and both G170D and T101I extracts, I found that a ~4-fold higher level of dTTP was required for telomerase to add a T at the +2 position in the T101I and G170D extracts relative to WT (Toogun *et al*., 2008); the K_m for nucleotide in the wild type extract was

found to be 797+/-56 nM where as it increased to 3314+/-347 nM in the T101I background. Thus, telomerase nucleotide affinity is altered in the absence of wild type Hsp82p.

Telomerase DNA binding is compromised in the G170D background

In addition to investigating telomerase nucleotide addition activity, our laboratory also examined whether Hsp82p has a role in assembling the telomerase DNA complex. A recent report has shown that human Hsp90 is required for human telomerase to effectively bind a telomeric DNA substrate (Keppler *et al.*, 2006). By exploiting the temperature sensitivity of the G170D yeast strain, fluorescence anisotropy on extracts prepared at either 30 °C or 37 °C found that only the 37°C G170D extract displayed a decreased DNA binding activity; the telomerase DNA binding affinity was reduced 5-fold in the G170D background for a K_d of ~1 nM to ~5 nM (Toogun *et al*., 2008). Importantly, telomerase DNA binding in the G170D 37°C extract was recovered upon the addition of purified Hsp82p (Toogun *et al*., 2008). These findings suggest that yeast Hsp90 supports at least two telomerase functions (DNA binding and extension) that are required for proper telomere maintenance. However, the affects of Hsp90 on telomere maintenance may not be limited to assisting in extension, as Hsp90 has also been implicated in affecting telomere capping components (Grandin and Charbonneau, 2001).

Cdc13 stimulates telomerase DNA extension activity

Hsp82 and Hsc82 are high-copy suppressors for the capping mutants *stn1-1* and *cdc13-1* yet in a wild type background elevated chaperone levels result in shortened

telomeric DNA (Grandin and Charbonneau, 2001). Neither the mechanism for the suppression nor the reduced telomere DNA length upon Hsp82-overexpression in wild type cells is understood. Thus, it appears that Hsp90 chaperones affect both capping and extending telomere components though the mechanistic contributions to the capping factors had yet to be revealed.

As an initial step I determined the Cdc13 effect on telomerase DNA extension activity in vitro. I anticipated that Cdc13 would inhibit telomerase function by competing for the DNA substrate, as observed for the human Cdc13 ortholog Pot1 (Kelleher *et al.*, 2005). To discriminate Cdc13 DNA binding-dependent effects I used DNA substrates with either 23- or 7-nucleotide 3'-overhangs in the telomerase extension assays. Prior biochemical studies indicated that Cdc13 requires a minimum of 11 single-stranded nucleotides to bind DNA (Mitton-Fry *et al.*, 2004). In accordance, I found that Cdc13 protein only bound to DNA- overhangs of 11 bases or longer (Figure 5A). Cdc13 was also unable to protect a short telomeric substrate (7 base overhang) from Exo-T nuclease activity, while it maintained a longer telomeric substrate (single stranded 30 base template) from degradation (Figure 5B). Unexpectedly, Cdc13 activated telomerase DNA extension activity independent of 3'-overhang DNA length (Figure 6A and B). The DNA products below the +1 position for the 23-base substrate are likely produced by a previously described telomerase-associated endonuclease activity (Niu *et al.*, 2000).

Cdc13 enhanced the extension of the 23-base substrate to a greater extent relative to the 7-nucleotide 3'-overhang DNA (~18- *vs.* ~9-fold) suggesting that DNA binding by Cdc13 positively contributed to telomerase activity. Nonetheless, the Cdc13 activation with a 7-base substrate suggested that Cdc13 could affect telomerase independent of

direct DNA binding. While stimulation by Cdc13 contrasts with the inhibitory effect of Pot1, it should be noted that Pot1 activates telomerase in conjunction with the telomeric protein Tpp1 (Wang *et al*., 2007). Hence, Cdc13 apparently functions in a manner comparable to the combined effects of Pot1 and Tpp1.

Cdc13, Stn1 and Ten1 form an unextendable telomere DNA Complex

In addition to Cdc13, Stn1 and Ten1 serve in telomere capping in vivo (Gilson and Geli, 2007). To determine whether Cdc13 can function with Stn1 and Ten1, I purified the proteins (Figure 7), and tested the effects of the proteins on telomerase activity. Ten1 alone caused inhibition of telomerase DNA-extension, while Stn1 alone activated telomerase DNA-extension on a 23-base 3'-overhang template (Figure 8). Cdc13 and Stn1 displayed a synergistic effect on telomerase DNA-extension, giving an increased activation state in comparison to either protein alone (Figure 8). In the presence of Cdc13 I observed a striking reduction in DNA extension with increasing Stn1/Ten1 levels using the 23-base 3'-overhang (Figure 8).

Given the established Hsp82 roles with capping and extending telomere components, I tested whether Hsp82 might affect Cdc13. As an initial test Hsp82 was titrated into DNA extension reactions containing a Cdc13/Stn1/Ten1-capped telomeric DNA substrate. In the absence of Supplementary Hsp82, Cdc13/Stn1/Ten1 suppressed the DNA extension activity below basal levels. However, in an Hsp82-dependent manner the extension activity increased, which indicates that Hsp82 is sufficient to switch the Cdc13/Stn1/Ten1-capping structure into a Cdc13/telomerase-extending complex (Figure 9).

Discussion

The presented work has important implications for both our understanding of Hsp90 and telomerase. In brief, yeast Hsp90 supports two telomerase functions (DNA binding and extension) that are required for proper telomere maintenance. Initial studies linking Hsp90 and telomerase advocated that the chaperone helped assemble the reverse transcriptase protein with the RNA template to form the core telomerase enzyme (Holt *et al*., 1999). Notably, the presented data uniquely demonstrate that Hsp82p operates with an enzyme after DNA binding. Perhaps the continued Hsp82p-association is required to maintain an open telomerase conformation that favors acceptance of free nucleotide and fosters the DNA extension activity. The ability of Hsp82p to serve a DNA bound enzyme might be a general function, as Hsp90 proteins have been found associated with additional DNA enzymes including other reverse transcriptases and the DNA helicase XPB, which is required for transcription initiation and DNA repair (Hu *et al*., 1997; Hu *et al*., 2004; Flom *et al*., 2005).

The multiple telomerase functions affected by Hsp82p parallels studies on the established Hsp90 client proteins steroid hormone receptors. Initially, Hsp90 was found as a component of the 9S untransformed steroid receptors for glucocorticoids (GR) or progesterones (PR) in which Hsp90 was required to maintain the high affinity hormone binding state and prevent aggregation of the untransformed receptors (Bailly *et al*., 1978; Sanchez *et al*., 1987; Pratt, 1993). In addition, Hsp90 was suggested to prevent DNA binding by the receptor through retention in the cytoplasm (Pratt, 1993). However, recent studies refute this last hypothesis and indicate that Hsp90 is found in the nucleus and

facilitates rather than inhibits GR DNA binding (Stavreva *et al*., 2004). In general, Hsp90 appears to support the DNA binding activities of heterologous transcription factors including p53, hypoxia-inducible factor 1 (HIF-1), aryl hydrocarbon receptor (AhR) and myogenic determination (MyoD) protein (Wilhelmsson *et al*., 1990; Shaknovich *et al*., 1992; Antonsson *et al*., 1995; Hur *et al*., 2004; Muller *et al*., 2004; Walerych *et al*., 2004). Hence, Hsp90 maintains DNA binding proteins in a state able to respond to activation signals (*e.g*., phosphorylation or hormone binding) and furthers the various pathways by promoting the DNA bound complex.

The presented data in conjunction with a prior study on the yeast p23 molecular chaperone Sba1p (Toogun *et al*., 2007) suggest that the Hsp90 and p23 chaperones jointly modulate telomerase DNA binding. In this model, Hsp82p and Sba1p cooperate to promote a rapid telomerase DNA binding cycle that would be highly beneficial for the addition of multiple telomeric DNA repeats during a single cell cycle—Hsp82p would promote assembly while Sba1p fosters disassembly. The coordinated chaperone actions on telomerase also parallel an intracellular receptor paradigm (Freeman and Yamamoto, 2001; Stavreva *et al*., 2004). Given the divergent nature of telomerase and intracellular hormone receptors, the common functional effects mediated by Hsp90 and p23 chaperones on these clients and recent proteomic studies, I suggest that these two molecular chaperones serve to maintain a wide variety of proteins in a dynamic state with DNA (Richter *et al*., 2007).

This work further provides a potential resolution to the apparent conundrum of how proteins with identical binding specificities coordinately function at a single site. Often DNA binding proteins form long-lived complexes with target DNAs in vitro that

would interfere with the assembly of other structures and telomere-protein assemblies are no exception (DeZwaan and Freeman, 2008). For example, a stable Cdc13-telomere association at the extreme 3' DNA end would block telomerase DNA binding yet Cdc13 binding at the extreme termini likely is required to protect the DNA from degradation in vivo. The data supports a model in which Hsp82 frees the DNA end of competing binding proteins without interfering with critical regulatory events.

In this model, telomerase recruitment to a telomere initiates through transient contacts between Cdc13 and the holoenzyme. To permit proper telomerase DNA association Hsp82 displaces proteins bound at the extreme 3' DNA end including Cdc13. Recent genetic studies highlight the potential importance of maintaining a dynamic telomere environment, as the peak of telomere association by Cdc13, Stn1 and Est1 all occur in S-phase (Puglisi *et al*., 2008; Taggart *et al*., 2002). Presumably, both capping (Stn1) and extending (Est1) proteins are telomere recruited each S-phase since not all telomeres are extended each cell cycle (Teixeira *et al*., 2004). Hence, depending upon the DNA length of a particular telomere a molecular choice is made to either permit or preclude telomerase function (*i.e.*, build an Est1-extending or Stn1-capping complex). If Hsp82 maintains Cdc13 in a dynamic DNA binding cycle, then any modifications (*e.g.*, phosphorylation) meant to guide a telomere to a distinct operative phase would be immediately incorporated.

Recent genetic studies suggest post-translational modifications mediated by the Tel1 (ATM homolog) and Cdk1 kinases are critical for proper telomere DNA maintenance (Tseng *et al*., 2007; Li *et al*., 2009). For example, Tel1 is recruited to critically short telomeric DNA tracts, which are preferentially elongated, and Cdc13 has

been shown to be a Tel1 target (Tseng *et al.*, 2007; Arneric *et al.*, 2007). In addition, Cdk1 phosphorylates Cdc13 and the modification appears to influence the preference between Stn1- and Est1-containing Cdc13 nucleated telomere complexes (Li *et al.*, 2009). Hence, depending upon the Cdc13 phosphorylation-state, telomeric DNA would be in a Cdc13-Stn1/Ten1 unextendable state or a Cdc13-Est1/telomerase extendable form. Dynamic Cdc13 action, mediated by Hsp82, would enable the telomere system to rapidly transition between the different structures as needed.

Prior genetic data suggests a role for Cdc13 with telomerase that is downstream of telomere nucleation. For example, the *cdc13-4* yeast have short but stable telomeric DNA despite an apparent capacity of Cdc13-4 to recruit telomerase to telomeres and protect the DNA from degradation (Meier *et al.*, 2001). While the effected residue (P235S) occurs within the amino-terminal domain, the mechanism for the telomere defect was not identified. However, the in vivo phenotype is consistent with a post-recruitment function for Cdc13 (Meier *et al.*, 2001). Intriguingly, the Cdc13-109 and Cdc13-231 mutants lead to over-elongated telomeric DNA despite a decline in DNA binding activity (Grandin *et al.*, 2000). If the sole positive function of Cdc13 was to recruit telomerase to a telomere, then a decline in DNA binding should result in shorter not longer telomeric DNA. Additionally, the hyper-elongation of telomeric DNA in the *cdc13-5* yeast is consistent with a post-recruitment role for Cdc13. The Cdc13-5 protein (N-domain) consists of the amino-terminus and DNA binding domain (Chandra *et al.*, 2001). In the absence of the Cdc13 carboxyl-terminal Stn1 interaction site the telomeric DNA should be vulnerable to nuclease attack. Yet, the telomeric DNA is not degraded in the *cdc13-5* yeast but rather is over-elongated (Chandra *et al.*, 2001). I suggest that, in addition to

recruiting telomerase, Cdc13-5 activates telomerase to not only hyper-extend the DNA but also to counter any potential DNA degradation. Of note, the stimulatory function is downstream from Cdc13-mediated recruitment since the effect is abrogated when combined with the Cdc13 point mutation *cdc13-2* (*i.e.*, *cdc13-2,5*) (Chandra *et al.*, 2001). Taken together, established genetic data support the contention that Cdc13 modulates the DNA-bound enzyme. I suggest that telomerase cofactors, including Cdc13, likely reengage the telomere protein assembly by tethering to the DNA-bound telomerase enzyme.

A chaperone-mediated protein dynamics model has been previously proposed for transcription pathways (Freeman and Yamamoto, 2001). In brief, molecular chaperones promote a dynamic action for transcription factors that is necessary to permit rapid functional recruitment of multiple coactivating complexes to a gene promoter. An important distinction, however, is the impact of the Hsp90 chaperone: Hsp90 promotes DNA binding by transcription factors but facilitates dissociation of Cdc13-DNA complexes. Perhaps the dual role for Hsp82 at telomeres provides an explanation for the difference. By exploiting the telomerase-associated cofactor Hsp82 to both support telomerase function and to clear the telomeric DNA of competing proteins, an elegant means to ensure telomeric DNA extension within the short working period allotted telomerase at the end of S phase is provided.

Traditionally Hsp90 has been viewed as a cytoplasmic molecular chaperone required for the late folding stages of signaling molecules (Wegele *et al.*, 2004). However, recent studies including high-throughput screens have identified a broad-range of potential nuclear client proteins (DeZwaan and Freeman, 2008). Given the impact of

Hsp90/Hsp82 both on telomeric and transcriptional targets, there appears to be a general cellular role for Hsp90 chaperones in controlling protein-DNA dynamics. In brief, multi-step pathways, including the telomere system, move forward through high affinity interactions between the low abundant proteins unique to that system (*e.g.*, Cdc13, telomerase, Stn1) while proper structure composition (*i.e.*, competitive interactions) along with efficient transitions between the different assemblies are mediated by transient, low affinity interactions with the highly abundant molecular chaperone

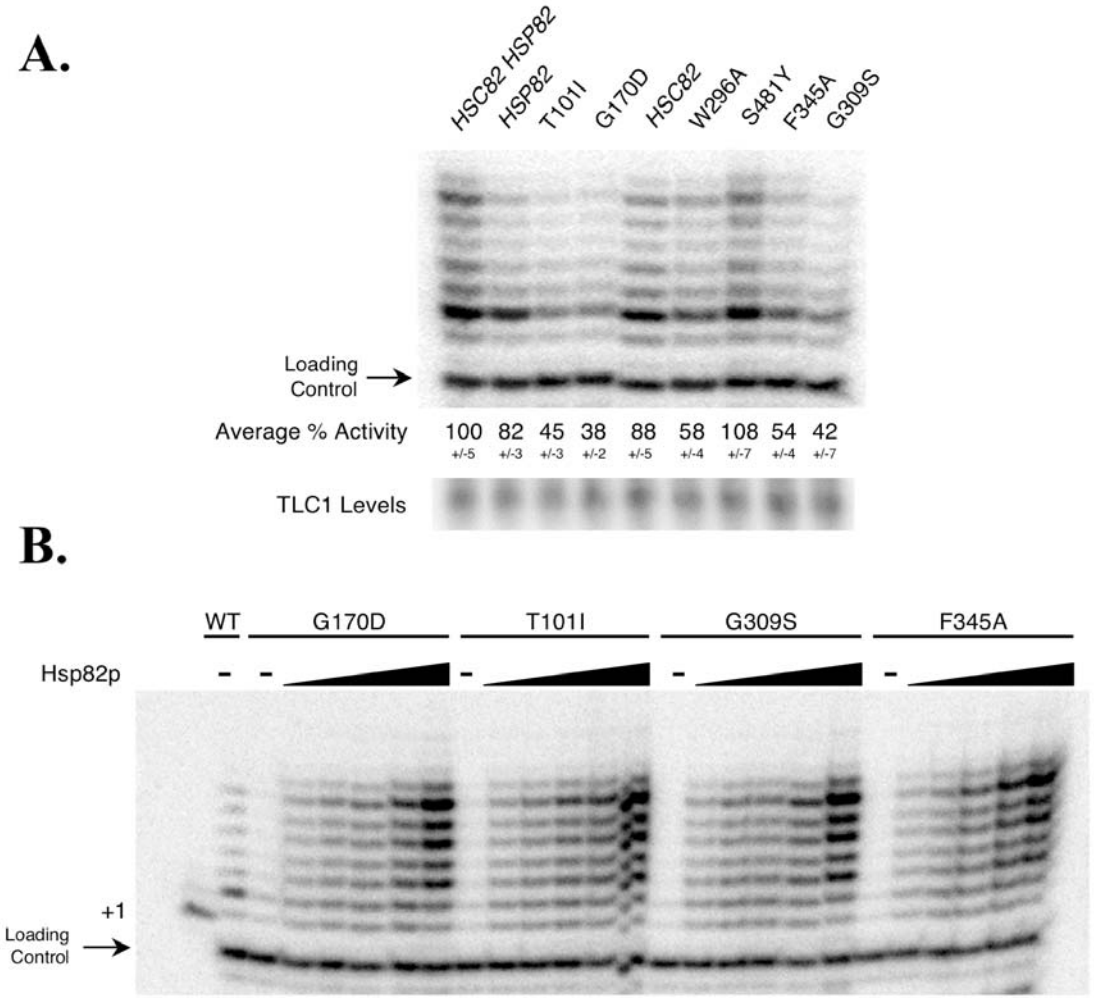

Figure 3. Telomerase DNA extension activity is Hsp82p-dependent in vitro. Telomerase-mediated extension of an immobilized 7-base single-stranded 3' overhang G-rich DNA substrate was examined using conventional DEAE telomerase extracts prepared from yeast expressing the indicated *hsc82* or *hsp82* alleles. (A.) The abilities of the indicated DEAE telomerase extracts to extend a telomeric DNA substrate in the presence of [a-^{32}P] dGTP and dTTP was determined. The extract prepared from the parental strain is *HSC82 HSP82* and those from the *hsp82* strains are clustered on the left (*HSP82*, T101I and G170D) while extracts made from the *hsc82* strains are shown on the right (*HSC82*, W296A, S481Y, F345A and G309S). The normalized percent DNA extension activities derived from 6 independent experiments for each extract is shown below each lane. The northern blot analysis showing the relative TLC1 RNA levels in each extract is shown below the DNA extension data; TLC1 levels were also checked by reverse transcription real time PCR analysis (data not shown). (B.) Purified Hsp82p was

(Figure 3 continued) sufficient to restore telomerase DNA extension activity in vitro. Recombinant, purified Hsp82p was titrated (0.2, 0.5, 1.0, 2.0, 4.0 mM) into the indicated telomerase extracts; endogenous cellular Hsp82p levels in yeast have been calculated to be ~17 mM (Picard, 2006). The extracts were prepared from the parental *HSC82 HSP82* (WT), *hsp82* allele (G170D or T101I) or *hsc82* allele (G309S or F345A) strains. All extension reactions contained equivalent levels of TLC1 RNA and a polynucleotide kinase end-labeled 27-base oligonucleotide was added prior to the precipitation of all the DNA extension products to serve as a loading control. The resolved extension products were visualized using a Molecular Dynamics PhosphorImager.

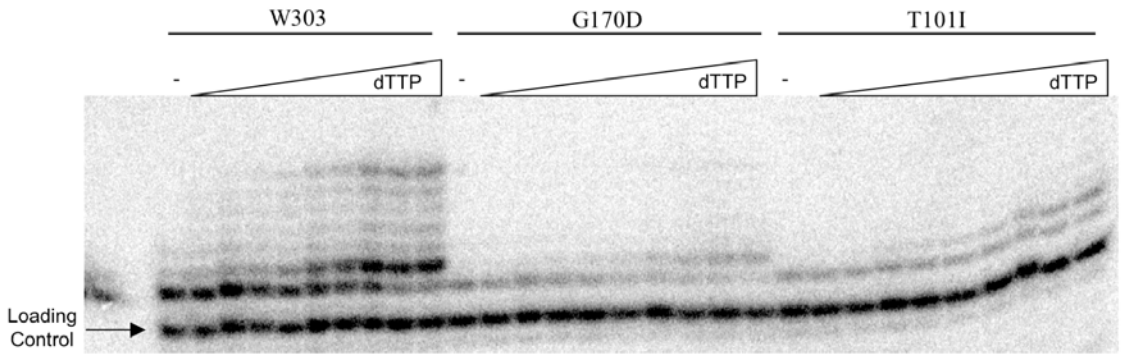

Figure 4. Hsp82p affects telomerase nucleotide affinity. The dTTP concentration needed for telomerase nucleotide addition in the presence and absence of wild type Hsp82p was determined using a dTTP titration. Telomerase extracts prepared from either yeast expressing wild type *HSP82* or the G170D and T101I alleles were incubated with [a-^{32}P] dGTP, 7-base 3'-overhang DNA substrate and various amounts of dTTP (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 or 64 μM).

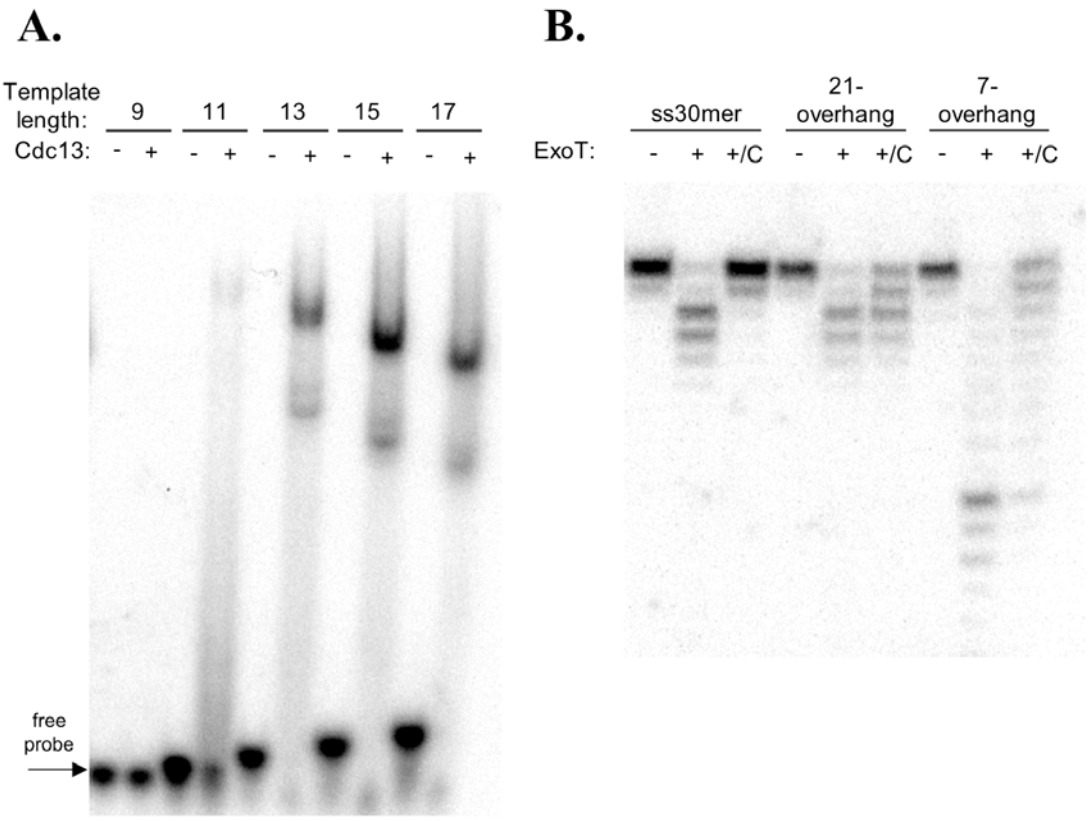

Figure 5. Cdc13 has the ability to associate with single-stranded DNA. (A.) Cdc13 needs at least 11 bases of single stranded DNA to form a complex. Electro-mobility shift assays were used to test the ability of Cdc13 to bind a series of single-stranded oligonucleotides (250pM). Template length ranged from 9 bases to 17 bases. (B.) Cdc13 protects the DNA end from nuclease digestion. Radio-labeled 30-nucleotide single-stranded telomeric (ss-30mer) DNA (250 pM) or hybrid single/double-stranded DNA substrates (250 pM) with either 21- or 7-base 3'-overhangs were treated with Exo-T nuclease alone or after incubation (2 min at room temperature) with full-length Cdc13 (C; 100 nM). All three substrates use the radio-labeled 30-nucleotide single-stranded DNA. The hybrid 3'-overhang substrates are formed with oligonucleotides complimentary to the 5' end of the 30-nucleotide single-stranded DNA (see Material and Methods). Following nuclease addition the reactions were incubated 12 min at 30°C, the reactions were stopped by the addition of formamide/NaOH loading buffer and boiling for 5 min, the samples were resolved on a 12% denaturing polyacrylamide gel, the polyacrylamide gel was dried and the products were visualized with a PhosphoImager. The 7-base 3'-overhang substrate likely displays no apparent protection by full-length Cdc13 since it is not bound by Cdc13.

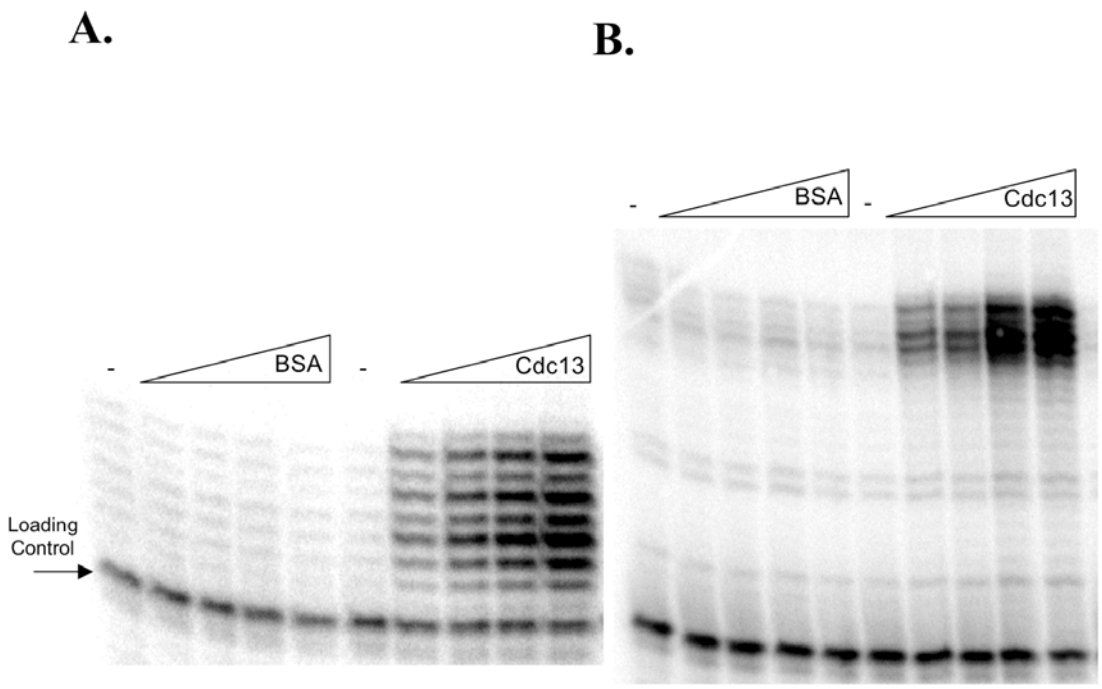

Figure 6. Cdc13 stimulates telomerase DNA extension activity independent of single-stranded 3'-overhang DNA length. The Cdc13 effect on telomerase-mediated DNA extension was determined using DNA substrates with either (A.) 7-or (B.) 23-nucleotide 3'-overhangs and a Cdc13 protein titration (50, 100, 250, 500 and 1000 nM), as marked. To control for possible non-specific protein affects the impact of BSA addition was tested. All extension reactions were supplemented with a loading control primer (arrow) prior to precipitation and electrophoretic resolution and the +1 position for each DNA substrates is marked.

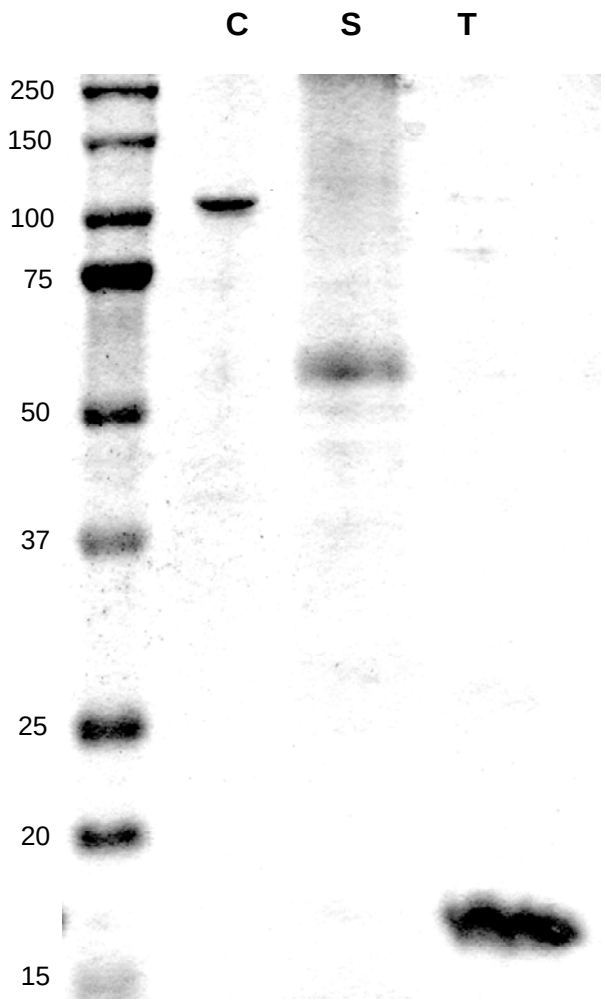

Figure 7. Purification of the "Capping" proteins. Recombinant purified (1 µg) ~103KDa Cdc13 full-length (**C**), 55KDa Stn1 (**S**) and 18KDa Ten1 (**T**) were resolved on a 12% SDS-PAGE and stained with Coomassie Blue.

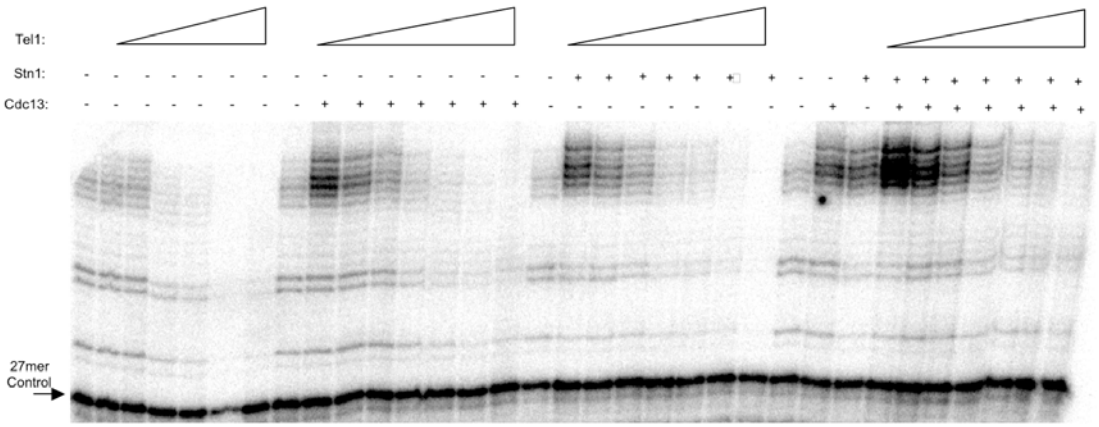

Figure 8. Stn1 and Ten1 cooperate with Cdc13 to form an unextendable telomere protein-DNA complex in vitro. The effect of a Ten1 protein titration (100, 250, 500, 1000, 2500 and 5000 nM) on telomerase DNA extension in the presence or absence of Cdc13 (50nM) and/or Stn1 (100nM) was determined using 23-base 3'-overhang DNA. For comparison, the activity of unsupplemented, Cdc13 supplemented and Stn1 supplemented telomerase extract is shown.

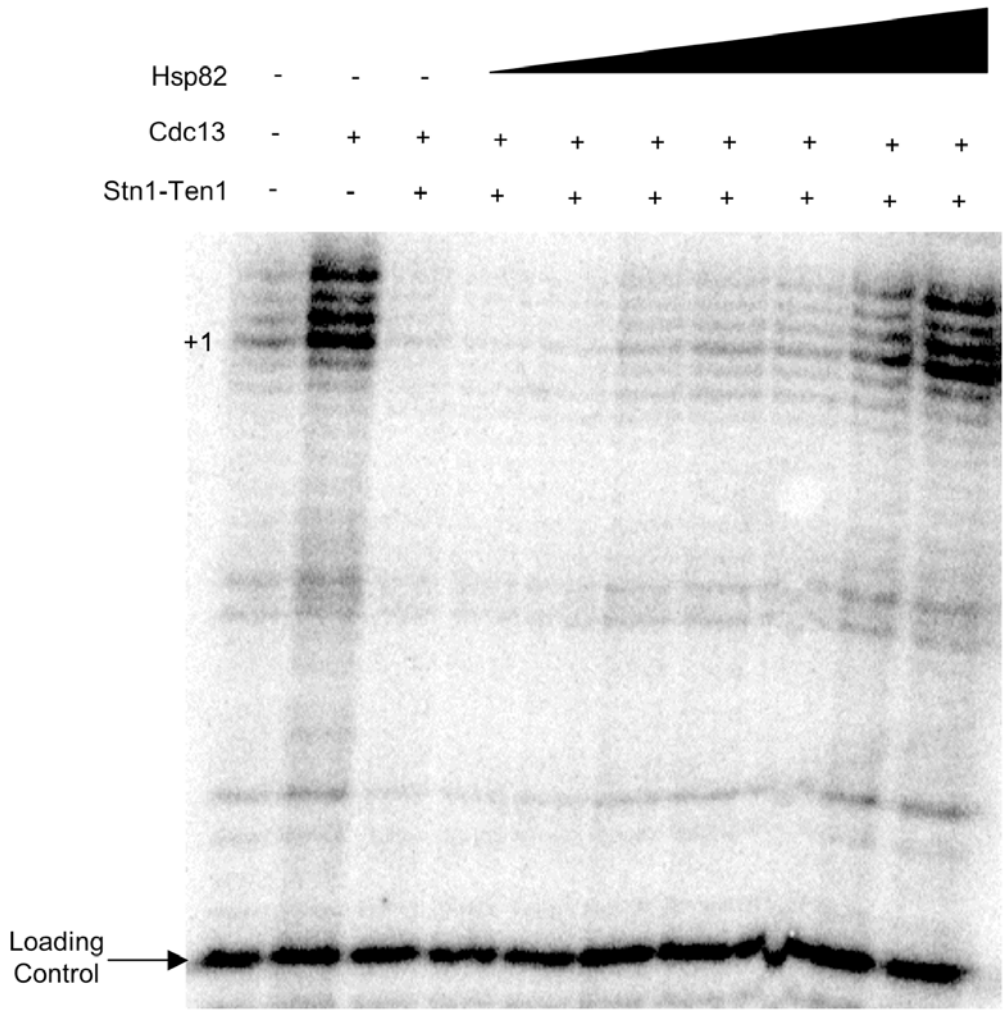

Figure 9. Hsp82 promotes the conversion of the Cdc13-capping structure into a Cdc13-extending complex. The ability of Hsp82 to convert an unextendable Cdc13/Stn1/Ten1-capped DNA complex into a telomerase accessible DNA structure was determined using a 23-base 3'-overhang DNA substrate, telomerase extract and an Hsp82 protein titration (0.25, 0.5, 1.0, 2.5, 5.9, 10.0 and 20.0 μM). The cellular Hsp82 concentration is 17μM (Picard, 2006).

CHAPTER 3*

BIOCHEMICAL CHARACTERIZATION OF EST1

Telomeres are nucleoprotein complexes found at chromosome ends that maintain DNA termini at an appropriate length to preserve genome stability and cell viability (Gilson and Geli, 2007). Most eukaryotes use the telomerase enzyme to sustain telomeric DNA. The telomerase core enzyme is comprised of a reverse transcriptase (Est2 in yeast) and an RNA template (TLC1) and the yeast holoenzyme includes the Est1 and Est3 cofactors. *EST1* was the first protein-encoding gene identified that was speculated to be a telomerase component, as *est1*Δ cells display the ever shorter telomere phenotype (Lundblad and Szostak, 1993). While Est1 is an established telomerase cofactor, its direct mechanistic contribution to telomerase activity had not been understood.

Genetic experiments suggest two potential Est1 roles with telomerase, as a bridging factor (Figure 2) coupling telomerase to the telomeric DNA binding protein Cdc13 during telomere recruitment and as an activator for telomerase enzymatic activity once the holoenzyme is telomere-bound. A seminal study, which exploited protein fusions between Est1, Cdc13 or telomerase, suggested an Est1 activation function since telomeric DNA is hyper-elongated in an Est1-dependent manner upon expression of a Cdc13-Est2 fusion protein (Evans and Lundblad, 1999). In addition, it was shown that covalent linkage of normally deleterious Est1 or Cdc13 mutants abrogates telomere defects implying that the mutations affect recruitment. Supporting a requisite Est1-

* Data presented in this chapter were originally published in **DeZwaan DC** and Freeman BC. (2009) The conserved Est1 protein stimulates telomerase DNA extension activity. *Proc. Natl. Acad. Sci. USA.*, 106:17337-42. I performed all of the presented experiments in this study.

Cdc13 interaction for proper telomerase regulation are reciprocal-charge mutants (*e.g.*, *est1-60* (K444E) suppresses *cdc13-2* (E252K)) that separately lead to telomere DNA shortening (Pennock *et al*., 2001). Since Est1-60 can bind telomerase in vivo, it was proposed that the K444E mutation disrupts Cdc13 association (Pennock *et al*., 2001; Bianchi and Shore, 2004). However, other studies suggest that the Cdc13-2 mutation does not disrupt Est1 interactions since wild-type Est1 interacts with Cdc13 and Cdc13-2 in vivo (Qi and Zakian, 2000; Taggart *et al*., 2002; Chan *et al*., 2008). Although in the absence of a direct biochemical assessment it is difficult to delineate the mechanistic effects of the various Est1 and Cdc13 mutations.

To modulate telomerase it has been speculated that Est1 relies on its two known activities, DNA and RNA binding. Prior biochemical studies demonstrate that yeast and human Est1 bind single-stranded G-rich DNA and apparently recognize RNA non-specifically in vitro (Vitra-Pearlman *et al*., 1996; Snow *et al*., 2003; Redon *et al*., 2007). In contrast to the in vitro work, in vivo studies indicate that yeast Est1 targets a bulged-stem loop in the TLC1 telomerase RNA (Livengood *et al*., 2002; Seto *et al*., 2002). The Est1-TLC1 interaction appears to be direct since it occurs in the absence of the Est2 protein in vivo (Steiner *et al*., 1996). Despite these reports, it was not apparent how Est1 modulates telomerase. I have attempted to understand the Est1 regulatory mechanism by investigating: 1) the Est1 impact on telomerase DNA extension activity; 2) the necessity of the TLC1 bulged-stem loop for Est1 RNA binding; 3) the Est1 DNA binding determinants; 4) the influence of Est1 nucleic acid binding on telomerase regulation; 5) the activities of various Est1 point mutants.

Est1 activates telomerase DNA extension activity

To study the mechanism of Est1 action the protein was purified to near homogeneity (Figure 10A and B). As an initial functional test, I titrated the Est1 protein into telomerase DNA extension reactions. I found that Est1 stimulated activity ~14-fold using telomerase extracts prepared from either *est1*Δ or parental cells (Figure 10C). As the DNA banding pattern is not reproducibly altered (*e.g.*, enhancement of longer products) upon Est1 addition but rather the relative product intensities are elevated, I suggest that Est1 increases the percent of active enzyme. Based upon the activation potential, Est1 displayed a high affinity for telomerase (K_d 3.5+/-1 nM in *est1*Δ and 27+/-4 nM in wild-type telomerase extract). The variance in Est1-telomerase affinities might result from the presence of Est1 protein, albeit limiting, within the telomerase extracts or from a telomerase structural difference. I suspect telomerase produced in the absence of Est1 is in a distinct conformation since the Est1 stimulatory effect is comparable using either wild-type and *est1*Δ telomerase extracts and even limiting Est1 amounts would diminish the fold-increase over the unsupplemented DNA extension activity. To understand the Est1-mechanism employed to modulate telomerase I examined the impact of Est1 properties on the stimulatory effect.

Est1 selectively binds to the TLC1 RNA bulged-stem loop

It has been suggested that a central bulged-stem loop within the TLC1 RNA is critical for telomerase regulation by Est1 (Livengood *et al.*, 2002; Seto *et al.*, 2002).

Therefore, I generated the Est1-TLC1 RNA binding site (bulged-stem loop fragment) and examined Est1 binding in vitro (Figure 11A). By electro-mobility shift analysis (EMSA) Est1 displayed a relatively high affinity for the bulged-stem loop RNA (K_d ~40 nM), albeit lower than its affinity for telomerase (Figure 10). The variation in RNA affinity relative to a prior report likely reflects the use of different RNA probes (Vitra-Pearlman *et al*., 1996). The Est1 RNA binding was dependent upon the bulged-stem loop structure, as a disrupted-stem RNA displayed no apparent interaction and a bulge-delete RNA had a decreased affinity (K_d ~300 nM). TLC1 RNA with compensatory mutations to reestablish the stem structure partially recovered the binding affinity (K_d ~150 nM) (Figure 11B). Thus, Est1 is dependent upon the bulged-stem loop structure to bind to the central TLC1 region and the in vitro binding determinants correlate well with prior in vivo studies (Seto *et al*., 2002).

The role of the TLC1 bulged-stem loop in conducting Est1 telomerase activation was determined by titrating Est1 protein into extension reactions with telomerase extracts prepared from yeast expressing full-length wild-type, stem-disrupt, bulge-delete or stem-compensatory TLC1 RNAs (Seto *et al*., 2002). The affect of the various RNA mutations on telomere length were initially established by southern blotting (Figure 12A). Telomere shortening was observed in both the bulge deletion and stem disruption mutants, while the stem compensatory mutant had telomere lengths comparable to wildtype TLC1 samples (Figure 12A). Unexpectedly, Est1 comparably activated the telomerase TLC1 derivatives (Figure 12B). Although the stem-disrupt telomerase displayed an ~2-fold Est1 affinity decrease, the effect is mild relative to the decline in binding to the stem-disrupt TLC1 RNA binding (Figure 11B). Based on these

differences, I suggest that the bulged-stem loop is one determinant in telomerase association but that other, likely protein-protein interactions between Est1 and Est2, are important for controlling telomerase activity.

Est1 binds single-stranded telomeric DNA

The initial Est1 biochemical characterization demonstrated that the protein preferentially binds DNAs with 3' single-stranded G-rich sequence (Vitra-Pearlman *et al.*,1996). I wished to extend these observations by delineating the DNA length requirements for Est1 binding. As an initial substrate I examined Est1 interactions with a 30-base, single-stranded G-rich oligonucleotide by EMSA and found that Est1 bound with an apparent K_d ~75 nM (Figure 13); no binding was observed using a C-rich 30-base primer (data not shown). Utilizing a series of single-stranded G-rich primers that varied from 15- to 30-bases I determined a minimal length requirement between 18 and 22 nucleotides for binding to a fully single-stranded DNA (Figure 14A).

I also tested the single-stranded DNA length requisite using a hybrid DNA substrate comprised of a single-stranded 3' end and a double-stranded 5' section. I found that Est1 bound well to 22- and 15-base 3'-overhang substrates but was unable to bind to shorter 3'-overhangs (Figure 14B). The ability of Est1 to bind hybrid DNAs with a 15-nucleotide overhang suggests that double-stranded DNA can be tolerated at the 5' end. Hence, depending upon the nature of the substrate (*i.e.*, fully single-stranded or hybrid) Est1 varied slightly in its DNA length requirements but still required single-stranded DNA for binding.

Next, I determined whether Est1 might bind RNA and DNA simultaneously, as a prior report had indicated (Vitra-Pearlman *et al.*, 1996). I added Est1 to reactions containing a radiolabeled DNA probe alone or with increasing amounts of unlabeled TLC1 RNA (Figure 15). If Est1 can concurrently bind DNA and RNA then a higher order, slower migrating complex would be expected; however, if binding were competitive then a decline in the Est1-DNA complex signal should occur. Since the Est1-DNA signal decreased with increasing TLC1 RNA levels (Figure 15), Est1 DNA and RNA binding is mutually exclusive. Contrary to the efficient competitive ability of TLC1 RNA for Est1 DNA interactions, I found that DNA competed ineffectively for RNA binding (data not shown). While the differential competition outcomes were unanticipated given the comparable Est1 binding affinities for RNA and DNA, the observed results likely show that TLC1 RNA is the preferred Est1 substrate. Minimally, my data indicate that telomerase regulation by Est1 involves only one bound nucleic acid.

Est1 telomerase activation is independent of Est1 DNA binding

To address whether Est1 exploits single-stranded DNA to effect telomerase action I performed extension assays using DNAs with relatively short or long single-stranded lengths. I found that Est1 equivalently up-regulated telomerase extension activity using substrates with 3'-overhangs of 7- to 24-nucleotides (Figure 16A). Importantly, the basal and Est1-stimulated DNA extension products from either substrate were telomerase-dependent since RNaseA treatment abolished activity (data not shown) (Prescott and Blackburn, 1997). The products below the +1 position for each DNA substrate are likely produced by a previously described telomerase-associated endonuclease activity (Niu *et*

al., 2000). Given the minimum 15-base requirement for Est1 DNA binding, it is unlikely that Est1 associates with the DNA during telomerase-mediated DNA lengthening.

To directly test whether Est1-DNA interactions influence telomerase regulation before assembly with telomeric DNA I performed order of addition experiments (Figure 16B). I preincubated Est1 or BSA with the DNA before adding telomerase (B), or telomerase with the DNA before adding the Est1/BSA (A). The reactions were initiated upon addition of the third component and free nucleotide. For the reactions where telomerase was preincubated with the DNA substrate, the immobilized DNA-bound complexes were washed to remove any unbound telomerase before initiating the reactions. Of note, similar reactions were performed with the 23-base DNA substrate where the DNA-bound complexes were washed to remove any unbound Est1/BSA/telomerase before initiating the reactions and comparable stimulatory effects were observed (data not shown). Since Est1 stimulated the DNA extension activity equivalently before or after telomerase DNA assembly, I suggest that Est1 tethers to DNA-bound telomerase and modulates the enzymatic activity independent of direct DNA binding by Est1.

Est1 point mutants display differential activities

Genetic analysis has generated a large Est1 point mutant collection that have been speculated to affect telomere maintenance by a number of means including altered Cdc13 association, defective TLC1 RNA interactions or declined telomeric DNA binding (Pennock *et al.*, 2001; Evans and Lundblad, 2002). In an attempt to better understand Est1 function, I have purified and characterized a select set including Est1-38, Est1-41,

Est1-42, Est1-49, Est1-50 and Est1-60. Prior in vivo analysis showed that Est1-38, Est1-41 and Est1-42 have lowered telomerase interactions but only altered telomeric DNA length slightly, Est1-49 and Est1-50 on the other hand displayed near wild-type telomerase binding but shortened telomeric DNA and Est1-60 does not support telomere DNA maintenance as it has been speculated to no longer interact with Cdc13 (Pennock *et al*., 2001; Evans and Lundblad, 2002).

I found that Est1-38, Est1-42, Est1-49 and Est1-50 had decreased RNA binding (~30, 62, 39 and 30% of wild type) and that Est1-38 and Est1-50 had declined DNA binding (~25 and 43% of wild type) and whereas the remaining mutants were functionally comparable to wild-type Est1 for nucleic acid interactions (Figure 17A and B). Notably, Est1-38, Est1-49 and Est1-50 comparably stimulated telomerase activity relative to wild-type Est1 (Figure 17C). In contrast, Est1-60 and Est1-41 had decreased (~34% and 38% of wild type, respectively) abilities to activate telomerase (Figure 17C); yet, neither showed an apparent nucleic acid binding defect. Thus, in agreement with the wild-type protein data, Est1 nucleic acid binding and telomerase activation potentials are non-correlative.

Since RNA and DNA binding did not have a significant influence on telomerase regulation by Est1 I suspected a direct protein-protein interaction between Est1 and Est2 was required. To determine if Est1 can interact with Est2 independent of TLC1 RNA, I synthesized Est2 using rabbit reticulolysate in the absence of TLC1 RNA. The Est2 protein was produced as an amino-terminal Protein A fusion protein to allow isolation with IgG sepharose (Zappulla *et al*., 2005). I found that Est1 can interact with Est2 independent of Tlc1 RNA (Figure 17D). The data agree with prior in vivo studies

showing that Est1 and Est2 interact in a *tlc1* null background and that human Est1A associates directly with the reverse transcriptase subunit in vitro (Bianchi *et al.*, 2004; Redon *et al*., 2007). However, the interaction is less efficient in the absence of Tlc1, which suggests that the Tlc1 RNA fosters assembly of the complex, as has been previously suggested (Livengood *et al*., 2002; Seto *et al*., 2002).

Next, I examined the interaction patterns for wild-type Est1, Est1-60 or Est1-41 (Figure 17D). I did not observe a significant change in association between the Est1 derivatives and Est2 alone. If, however, I synthesized the Est2 in the presence of TLC1 and then performed the pull-down I observed an enhanced Est1-60 association (~2.5-fold increase) and a declined Est1-41 interaction (~40%) relative to wild-type Est1 (Figure 17E). The reduced telomerase association by Est1-41 is comparable to the decreased in vivo interactions previously observed for Est1-41 and telomerase (Evans and Lundblad, 2002). Since neither Est1 mutant displayed an inherent RNA binding defect (Figure 17), I suggest that TLC1 binding has an allosteric effect on either or both Est1 and Est2, which alters Est1 interactions with the core enzyme.

To determine the relative telomere occupancy levels of Est1, Est1-41 and Est1-60 in vivo a chromatin immunoprecipitation assay was employed (DeZwaan and Freeman, 2009). A similar telomere interaction pattern was found for the Est1 derivatives as observed for RNA binding—Est1-41 levels were reduced but Est1-60 and wild-type Est1 occupancies were comparable (DeZwaan and Freeman, 2009). While the in vitro and in vivo data are consistent, the Est1-60 telomere binding was unexpected since prior genetic work predicted that Est1-60 (K444E) would not associate due to a disrupted salt-bridge interaction with Cdc13 (Pennock *et al*., 2001). Based upon the presented data, I suggest

that the reported telomeric DNA shortening in the *est1-60* yeast involves a defect in telomerase stimulation, in addition to a perturbed interaction between Cdc13 and Est1 that is essential for proper telomerase function at a telomere. To understand if the Est1-60 mutation affects functional interactions involving Cdc13 and telomerase our laboratory investigated the combinatorial effects of Est1 and Cdc13 on telomerase DNA extension activity.

Prior genetic work showed that the telomere DNA length defect in the *est1-60* background is alleviated by the second-site suppressor mutation *cdc13-2* (Pennock *et al.*, 2001). Therefore, if the inability of Est1-60 to fully stimulate telomerase in vitro is related to the in vivo telomere phenotype then inclusion of the Cdc13-2 mutant should reestablish proper telomerase up-regulation with Est1-60 in vitro. To determine whether interactions between Est1 and Cdc13 impact telomerase our laboratory tested the individual and combined effects of purified Est1 and Cdc13 proteins on telomerase DNA extension activity in vitro. Of note, it was found that Cdc13 was capable of stimulating telomerase DNA extension activity independent of Est1 and I have followed this observation in a separate study (DeZwaan *et al.*, 2009). The combination of Est1 and Cdc13 led to up-regulation of telomerase isolated from wild-type yeast (DeZwaan and Freeman, 2009). While the cellular level of Est1 (a telomerase holoenzyme subunit) has not been determined, if we assume an amount comparable to the TLC1 RNA core subunit than the Est1 cellular concentration would be in the lower nanomolar range (Modzy and Cech, 2006). Significantly, the combinatorial Est1-Cdc13 effect was not apparent when one of the wild-type proteins was substituted with a mutant derivative. Yet, in the presence of both Est1-60 and Cdc13-2 telomerase activation was apparent (DeZwaan and

Freeman, 2009). Since the combinatorial Est1-Cdc13 effect was apparent using either a short (7-base) or long (23-base) 3'-overhang substrate, I suggest that DNA binding by Est1 and Cdc13 is not imperative to stimulate the DNA-bound telomerase enzyme. Hence, in a manner paralleling the in vivo phenotypes the Est1-60 and Cdc13-2 mutants compensate and reestablish activity comparable to the wild-type condition. While the interaction between Est1 and Cdc13 might influence a number of telomere events, our data demonstrate that the association is minimally required to properly control the enzymatic function of telomerase.

Discussion

Est1 is a fundamental telomere DNA maintenance factor that is conserved from yeast to human (Snow *et al.*, 2003; Reichenbach *et al*., 2003). Genetic experiments in yeast have suggested an Est1-role in both recruiting and activating telomerase (Evans and Lundblad, 1999). Yet evidence demonstrating a direct Est1 regulatory function with telomerase had been lacking. Here I show that purified Est1 protein stimulates telomerase DNA extension activity in vitro, which parallels recent work with the human factors (Redon *et al.*, 2007). However, in contrast to prior suggestions, the direct Est1 regulatory mechanism does not significantly rely on either telomeric DNA or TLC1 RNA binding.

While Est1 TLC1 interactions are dependent upon the bulged-stem loop structure both in vitro and in vivo (Figure 11B; Livengood *et al*., 2002; Seto *et al*., 2002) alteration of the bulged-stem loop only had a minor effect on telomerase stimulation in vitro suggesting Est1 recognizes TLC1 but the association is not a major activation-

determinant. Given the requisite for the TLC1 bulged-stem loop for telomerase binding in vivo, why isn't there a more significant effect in vitro? Perhaps the RNA interaction has an important role during telomerase holoenzyme assembly that is essential for efficient telomerase action in vivo but is dispensable in vitro. Presumably holoenzyme assembly is required for efficient telomere-recruitment in vivo; the presented in vitro assays likely are not impacted by recruitment events. The Est1 association with TLC1 RNA in the absence of the Est2 protein indicates that the Est1-TLC1 interaction might represent the first step in holoenzyme assembly in vivo (Steiner *et al.*, 1996).

In addition to RNA interactions, it has been suggested that single-stranded DNA binding by Est1 might contribute to telomerase modulation (Vitra-Pearlman *et al*.,1996). However, Est1 does not appear to rely on DNA interactions to affect telomerase in vitro (Figure 16). Perhaps the Est1 DNA binding ability provides a means to telomere-associate independent of telomerase but once telomerase arrives Est1 switches ligands and binds to TLC1—comparable DNA/RNA swapping has been previously postulated for other factors (Suswam *et al.*, 2005). Although the necessity of the Replication Protein A (RPA) for Est1 telomere loading suggests Est1 DNA binding activity is insufficient for telomere interactions (Schramke *et al.*, 2004). Alternatively Est1 DNA binding might be important for other non-telomeric cellular functions.

In my telomerase regulatory model, Est1 joins the core enzyme by selectively binding the TLC1 bulged-stem RNA loop—assembly of the holoenzyme prior to telomere association is requisite for downstream control events including telomerase up-regulation (Livengood *et al*., 2002; Seto *et al*., 2002; Chan *et al*., 2008). Once the holoenzyme engages telomeric DNA the contacts between Est1 and Est2 are critical for

efficacious telomerase DNA extension activity. In addition to Est1, telomerase regulation in vivo requires Cdc13—minimally interactions between Est1 and Cdc13 are used to modulate the holoenzyme. Though it had been suggested that a salt-bridge linkage between Est1 and Cdc13 is required for Est1 nucleation at a telomere, it appears that this is not the case. Prior studies had interpreted the telomeric DNA shortening in the *est1-60* background as an inability to recruit Est1 to the telomere and the phenotype suppression in *est1-60 cdc13-2* yeast further supported this notion (Pennock *et al.*, 2001). However, the Est1-60 protein displayed no apparent telomere recruitment defect (Figure 17). In conjunction with previous reports showing that Est1 interacts at telomeres in both wild-type and *cdc13-2* yeast (Taggart *et al.*, 2002; Chan *et al*., 2008), the necessity for the Est1(K444)-Cdc13(E252) salt-bridge for Est1 telomere recruitment is unlikely. While interactions between Est1 and Cdc13 might be essential for stabilizing telomerase at a telomere in vivo, our studies have not addressed this potential function.

Minimally, the Est1-Cdc13 salt-bridge is important for regulating telomerase enzymatic activity. While the absolute Est1 requisite for telomere DNA maintenance in vivo can be bypassed using a Cdc13-Est2 fusion protein, the significant reduction in telomeric DNA lengthening upon *est1* deletion in the Cdc13-Est2 fusion background supports an Est1 role independent of telomere recruitment (Evans and Lundblad, 1999). The covalent linkage between the telomerase Est2 protein subunit and Cdc13 likely bypasses any potential telomere stabilization role by Est1. The inability of Est1-60 to stimulate telomerase in vitro despite its capacity to interact with Est2 suggests that the mutant does not properly shift telomerase to a stimulated state (Figure 17). Perhaps in the presence of the Cdc13-2 protein, which suppresses the Est1-60 defect, the

conformational switch in telomerase occurs. I suggest the *cdc13-2* and *est1-60* mutations separately interfere with a critical contact point between the two proteins that is necessary for telomerase stimulation and for potentially stabilizing telomerase to a telomere. Presumably, in the presence of both protein mutants the charge-swap reestablishes the Est1-Cdc13 interaction and permits proper telomerase control. Further studies will be necessary to delineate the potential structural changes that occur within the various telomere-binding proteins to mediate these regulatory steps. Nevertheless, the presented data provide a novel understanding of Est1-telomerase control.

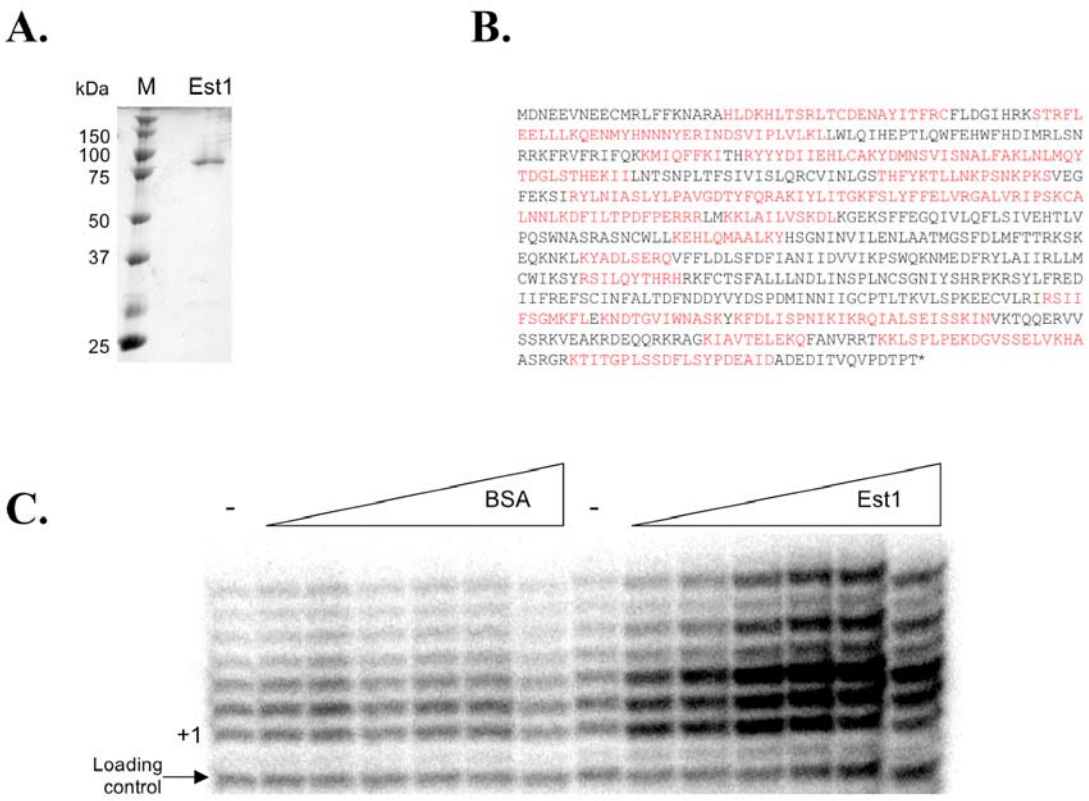

Figure 10. Purified Est1 enhances telomerase DNA extension activity in vitro. (A.) Recombinant Est1 (1 mg) was resolved on a 12% SDS-PAGE and stained with Coomassie Blue. (B.) The identity of the Est1 protein was confirmed by mass spectrometry analysis. The masses of peptides produced by the tryptic digestion of soluble Est1 protein were measured by MALDI TOF/TOF mass spectrometry. The amino acid Est1 sequence is shown with all of the identified peptides highlighted in red. For an example of the raw data please see file:///Users/bfreeman/Documents/Microsoft%20User%20Data/Saved%20Attachments/Freeman%203–1-2007.htm. (C.) The Est1 effect on telomerase-mediated DNA extension was tested using extracts prepared from *est1*D yeast with an immobilized 7-base 3'-overhang DNA substrate. The DNA extension reactions were supplemented with BSA or Est1 (1, 2.5, 5, 10, 25, 50 nM), as marked. To serve as a loading control an end-labeled 27-base primer was added prior to precipitation of the extension products. The positions of the loading control primer and +1 extension product are marked.

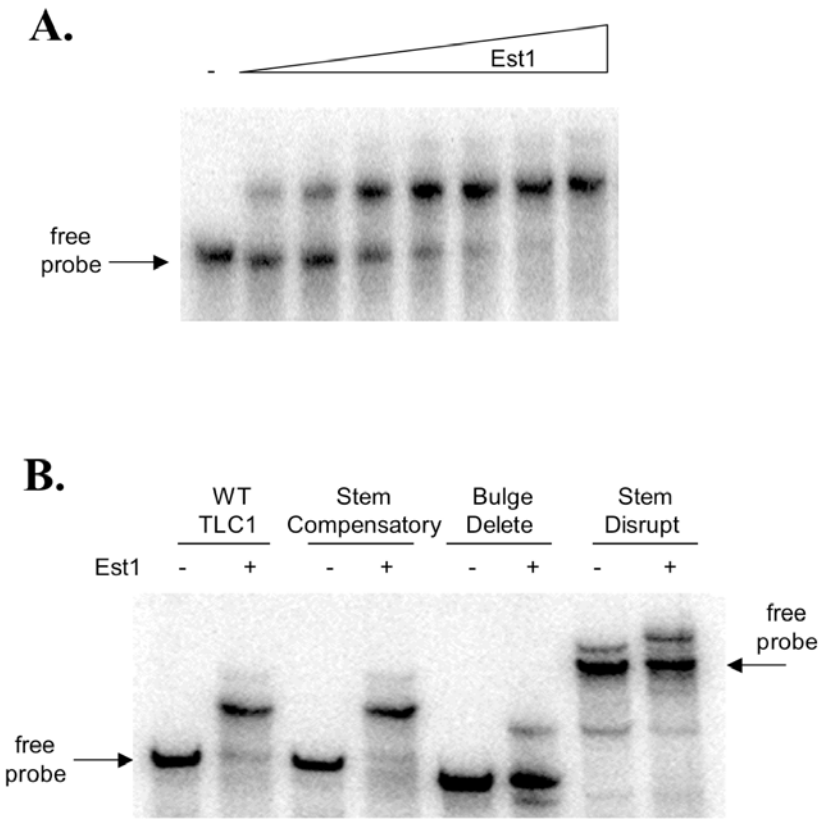

Figure 11. Est1 RNA binding is dependent upon the central TLC1 bulged-stem loop. (A.) To assess the Est1 RNA binding activity EMSA was used in conjunction with a radiolabeled TLC1 RNA probe. To measure the interaction affinity between Est1 and TLC1 the wild-type bulged-stem loop RNA (250 pM) was incubated alone or with varying Est1 concentrations (30, 60, 120, 240, 480, 960, 1440 nM). All binding reactions were resolved by native polyacrylamide electrophoresis. The arrows mark free probe locations. (B.) The Est1 RNA binding specificity was determined using established TLC1 RNAs that included stem disruption, bulge deletion and stem compensatory mutants (Seto *et al*., 2002). The slower migration for the stem-disruption RNA indicates that it is in a distinct conformation. The ability of Est1 (480 nM) to bind to each TLC1 derivative (250 pM) was determined by RNA EMSA. The asterisk marks the position of a minor slower migrating, distinctly folded RNA that occurs with the stem disrupt RNA. Though the migration of minor species reproducibly slows further following incubation with Est1, the change in migration is not dependent upon a stable Est1 interaction as it is apparent even after degradation of the Est1 following protease addition (data not shown). Hence, the slight migration shift in the minor RNA species present in the stem disrupt experiment does not result from a stable association with Est1.

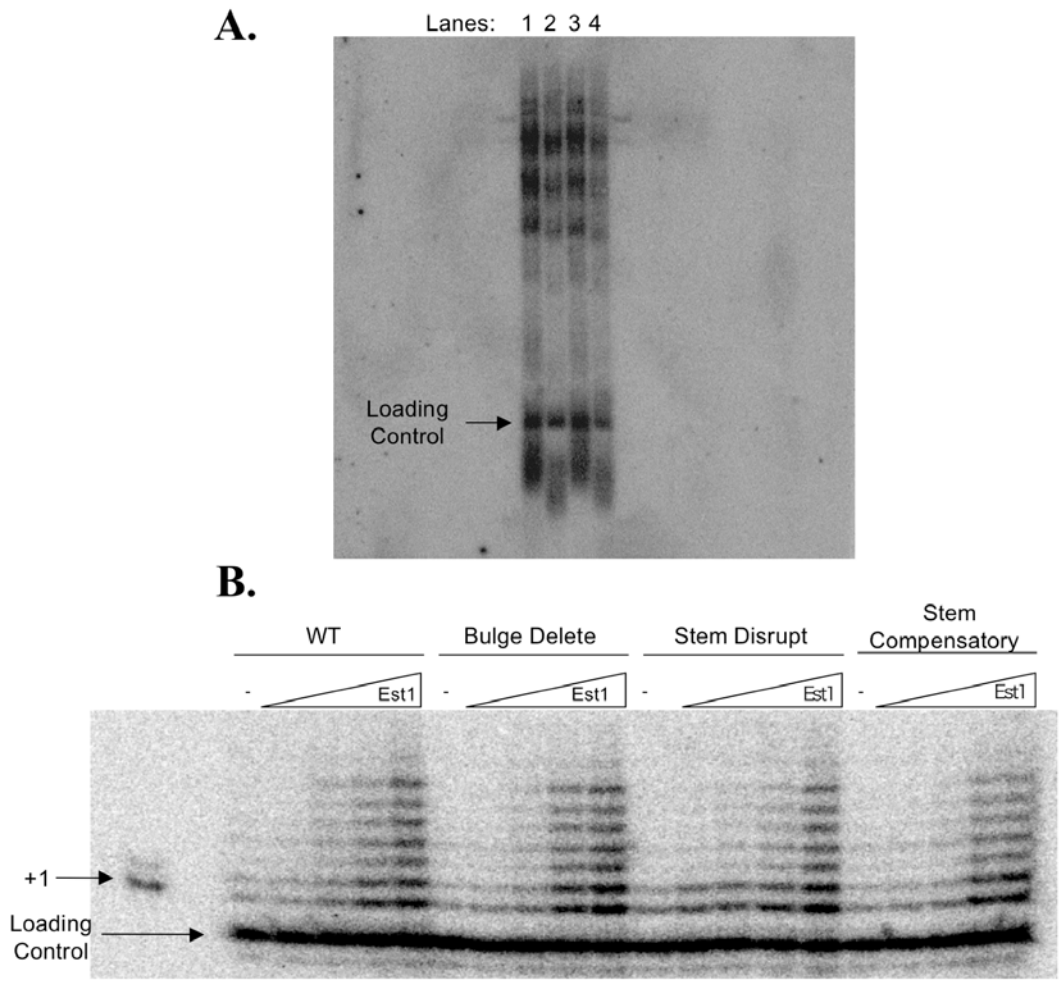

Figure 12. Est1 does not use its RNA-binding ability to enhance telomerase extension. (A.) The affects that the TLC1 mutants have on telomere length were tested by southern blotting. Lane 1.) Wildtype, 2.) Bulge Delete, 3.) Stem Compensatory and 4.) Stem Disruption. The arrow marks a "Cech Chromosome IV" probe used as a migration control, displaying that the changed migration was due to telomere shortening and not a whole chromosome problem. (B.) The Est1 effect on telomerase enzymes with the full-length TLC1 bulged-stem loop derivatives was tested using in vitro DNA extension assays and a 7-base 3'-overhang DNA substrate. Reactions were supplemented with increasing Est1 amounts (1, 10, 100 or 200 nM), as designated.

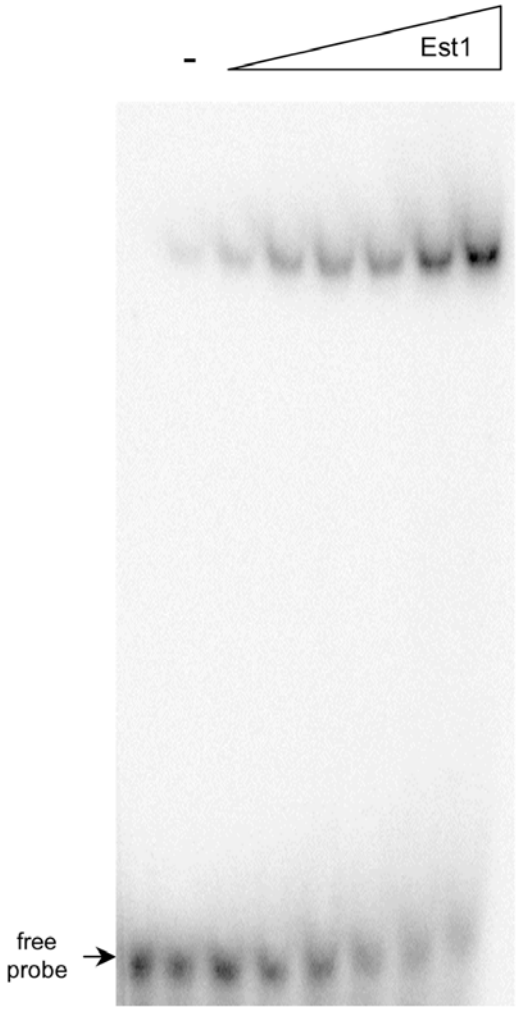

Figure 13. Est1 binds single-stranded G-rich DNA in a DNA length-dependent manner. The Est1 binding affinity for single-stranded G-rich DNA was determined by EMSA. The binding reactions contained a PNK end-labeled 30-nucleotide G-rich primer (50 pM) alone or with increasing Est1 levels (5, 10, 25, 50, 75, 100, and200 nM). Reactions were carried out at room temperature in TMG-30 buffer supplemented with BSA 200 g/mL, 200 mg/mL poly[d(I-C)]. The free probe is indicated with an arrow.

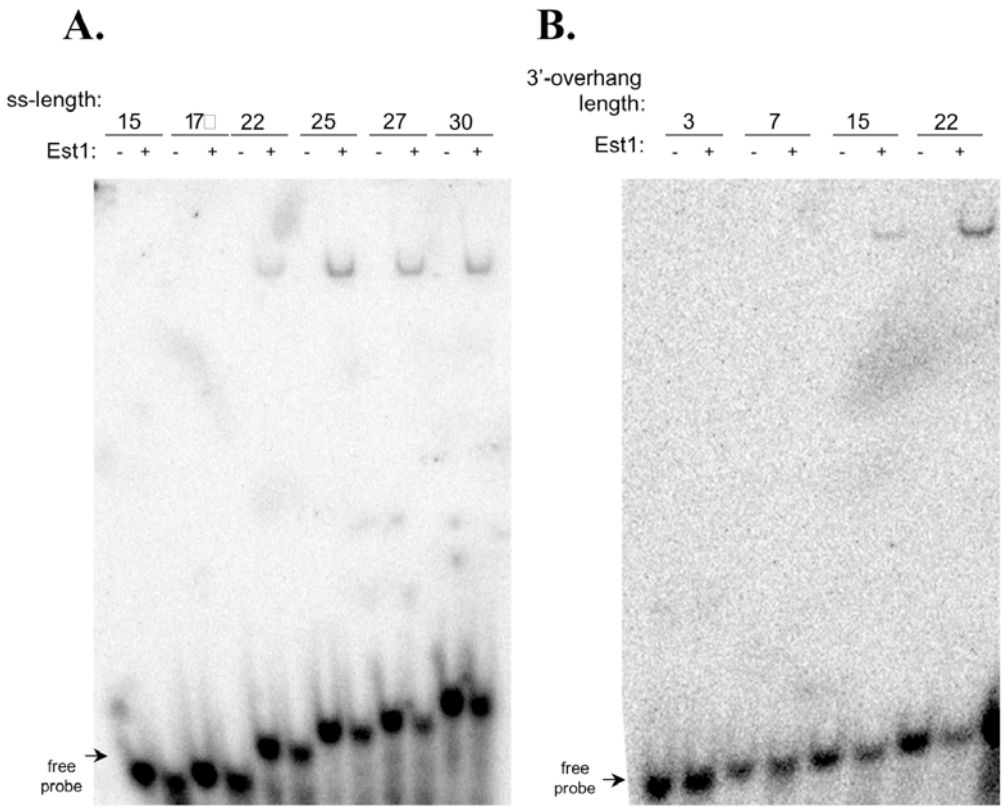

Figure 14. Est1 binds both single-stranded and hybrid/single-stranded DNA. (A.) The single-stranded DNA length required for Est1 (100 nM) binding was determined using primers (250 pM) with the indicated lengths. (B.) The ability of Est1 (100 nM) to bind to hybrid DNAs (250 pM) with single/double-stranded sections was determined using substrates with varying 3'-overhang lengths, as specified. The DNA electromobility shift assays contained the indicated Est1 protein amounts in TMG-30 buffer supplemented with BSA 200 g/mL, 200 mg/mL poly[d(I-C)], and end-radiolabeled oligonucleotide. The single-stranded primers were based on GTGGGTGTGTGTGTG (15mer) with additions to the 5' end accounting for the longer sequences—GG (17mer), TGTGTGG (22mer), GTGTGTGTGG (25mer), TGGTGTGTGTGG (27mer) or GGGTGGTGTGTGTGG (30mer). To create the hybrid single-/double-stranded substrates shorter complimentary oligonucleotides were annealed to the 5 end of the G-rich 30mer. The generated 3 -overhangs varied in length from 3 bases (ACCCACACACACCCACACACACCACC), 7 bases (ACACACACCCACACACACCACCC), 15 bases (CCACACACACCACCC), or 22 bases (CACCACCC).

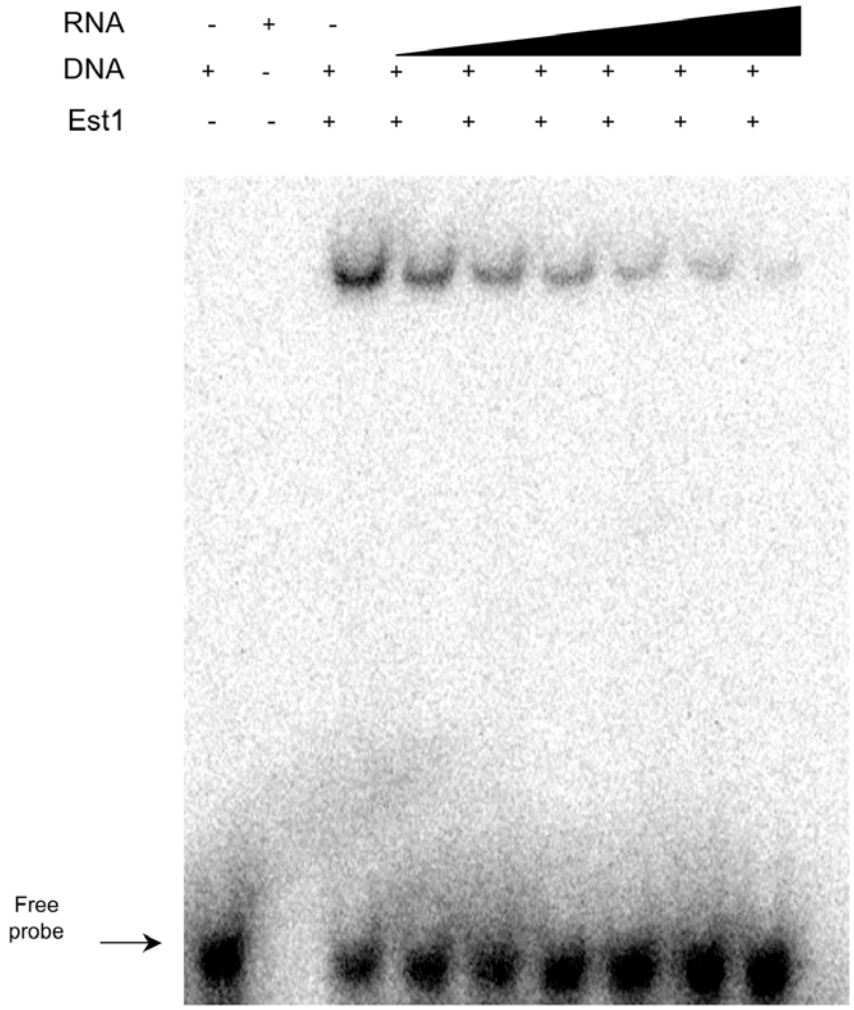

Figure 15. Est1 nucleic acid binding is mutually exclusive. An RNA/DNA competition assay was used to determine if the Est1 DNA and RNA binding activities are exclusive. Est1 (100 nM) was incubated with radiolabeled 30-base single-stranded DNA primer (250 pM) alone or with increasing amounts of unlabeled TLC1 RNA(0.25, 0.5, 1.0, 2.0, 4.0, or 8.0 nM), as marked. After a 20 minute incubation at 25 °C, the samples were resolved on a 6% native polyacrylamide gel in 1 XGTG buffer (90 mM Tris, 29 mM taurine, and 0.7 mM EDTA), the polyacrylamide gel was dried and the products were visualized with a PhosphoImager.

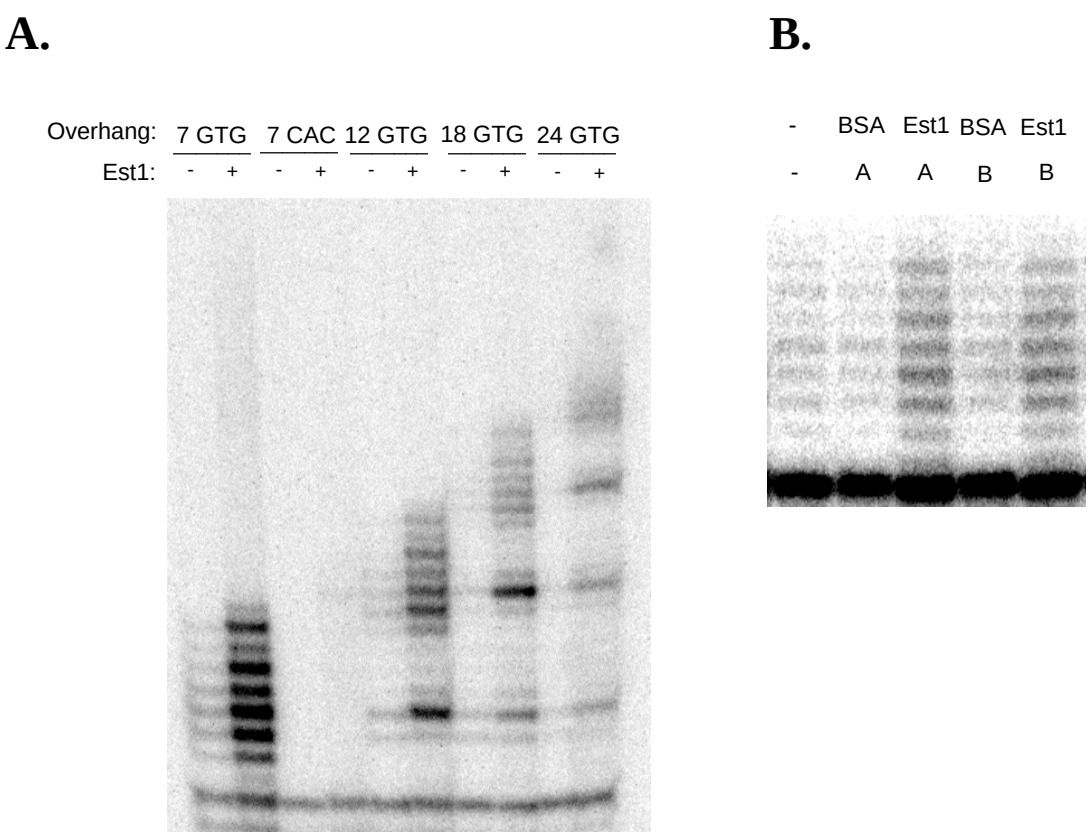

Figure 16. Est1 up-regulates the telomerase DNA extension activity independent of DNA interactions. (A.) The capacity of Est1 (50 nM) to stimulate telomerase-mediated DNA extension using substrates with various 3'-overhang lengths was determined. To demonstrate that the DNA extension products were telomerase-dependent the telomerase extracts were tested using a DNA substrate with a C-rich 3'-overhang. (B.) The Est1 ability to affect free or DNA-bound telomerase was determined by altering the order of addition for Est1/BSA (50 nM), and the telomerase extract on a 7-base DNA substrate. The (A) indicates that Est1/BSA was added to reactions after telomerase had been pre-incubated with the DNA. The (B) indicates that Est1/BSA was pre-incubated with the DNA before the addition of telomerase to the reaction. The positions of the loading control primer and the various +1 DNA extension products are marked.

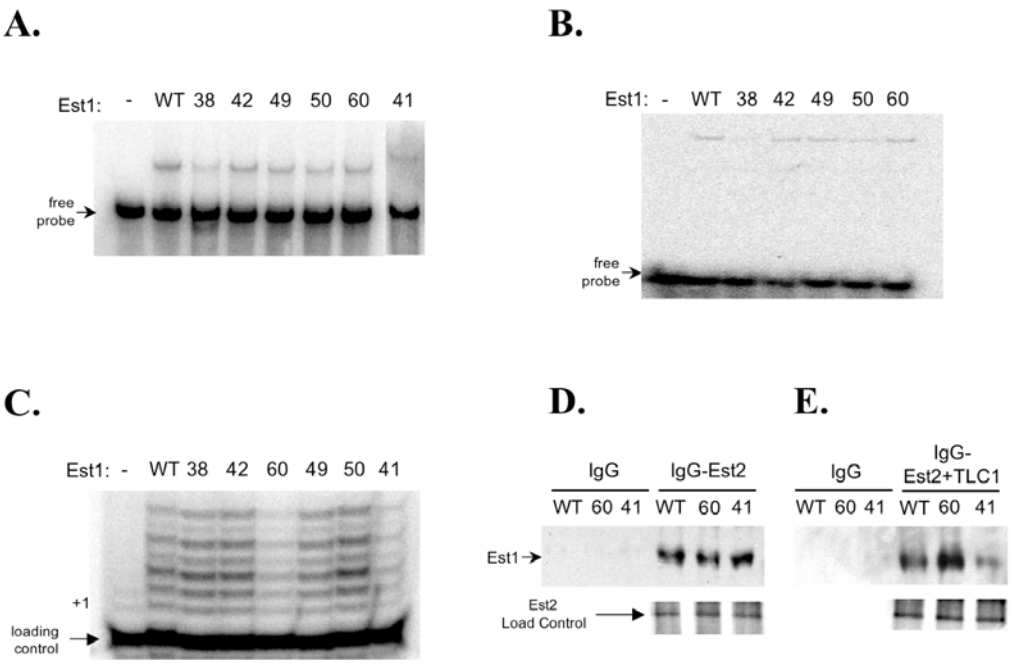

Figure 17. Defects in nucleic acid binding and telomerase stimulation are non-correlative for Est1 derivatives. The Est1 point mutants Est1-38, Est1-41, Est1-42, Est1-49, Est1-50 and Est1-60 were purified to near homogeneity and characterized. (A.) The RNA binding activity of each derivative (100 nM) was determined by EMSA using a radiolabeled TLC1 RNA (250 pM). (B.) The DNA binding activity of each derivative (100 nM) was determined by EMSA using a radiolabeled single stranded G-rich 30 base oligonucleotide (250 pM). (C.) The ability of the various Est1 proteins (50 nM) to enhance telomerase DNA extension activity was examined. The positions of +1 and the loading control primer are specified. (D.) The capacity of Est1 to interact with Est2 independent of TLC1 RNA was examined in vitro. Est1 (500 ng) was added to either IgG sepharose alone or IgG sepharose preincubated with either ProA-Est2 + Tlc1 or ProA-Est2. Est1 loading (20% input) control data is shown. (E.) The abilities of wild-type Est1, Est1-60 and Est1-41 to interact with Est2 in the absence or presence of TLC1 RNA were determined using Est2 protein immobilized on IgG sepharose. Representative data from 3 independent experiments is shown. For the pull-down experiments the Est1 proteins were visualized by western blot analysis using an antibody directed against the His_6-tag. Est2 loading control data is shown where the [^{35}S]Met-labeled Est2 translation products were resolved by SDS-PAGE and visualized using a PhosphoImager.

CHAPTER 4*

DISCUSSION

The telomere protein system has a variety of activities, including protecting, extending and replicating the terminal DNA in order to maintain the chromosomal ends (Figure 1). Although telomeres from all eukaryotes perform similar functions, detailed studies in budding yeast make it a particularly useful model system. During each cell cycle the telomere protein composition transitions from one complex to the next depending upon phase and marked need. For example, at telomeres with relatively short telomeric DNA tracts, the organization will fluctuate from an M-phase capping complex (minimally Cdc13–Stn1–Ten1) to a G1 capping structure that includes YKu70–YKu80-stabilized telomerase (core subunits) to the DNA replication machinery in S-phase to a DNA resectioning assemblage (involving the MRX complex and the ExoI and SaeI nucleases) that degrades a segment of the C strand in late S phase to a DNA-extending structure (telomerase–Est3–Est1–Cdc13) for the G-rich 3'-overhang strand to a DNA polymerase a complex (Pol1, Pol12 and primase) that fills in the complementary C-strand during S/G2 and then returns to the capping structure found in M phase (Figure 1). In addition to this route, the telomere structure can remain capped throughout the cell cycle. The decision to follow an extendable or unextendable path is determined, in part, through a counting mechanism involving the number of double-stranded telomeric DNA motifs (i.e. length of terminal DNA), the Rap1–Rif1–Rif2 protein complex, and the telomere-

* Figures in this chapter were modified from **DeZwaan DC** and Freeman BC. (2010) HSP90 manages the ends. *Trends Biochem. Sci.,* 35: 384-91.

associated kinases Tel1 and Mec1 (Shore and Bianchi, 2009). Intriguingly, Hsp82 interacts with at least one operational component required for each of these telomere events (highlighted in red in Figure 1). But what functional role does Hsp82 have with these various factors? The telomere system is confronted with difficulties that might be alleviated with the assistance of a molecular chaperone. Specifically, telomeres face significant protein motility challenges; there is a need to transition between multiple structures and so individual telomere assemblies require mobility to function efficiently (e.g. telomere lengthening requires telomerase repositioning) and the system must avoid potential competitive DNA-binding events that would otherwise sterically interfere with function (e.g. Cdc13, Est1 (ever shorter telomeres), MRX, Stn1– Ten1 and telomerase are all single-stranded, G-rich DNAbinding proteins) (Gilson and Geli, 2007; Shore and Bianchi, 2009). Although there are a number of possible explanations to account for the various challenges, I favor a model in which the telomere environment is maintained in a dynamic state by the HSP90 machinery. If telomeres were governed by dynamic remodeling, then the individual components would exchange rapidly, fostering efficient transitions between complexes and avoiding competitive binding events through transient DNA interactions.

Cdc13 plays a central role in yeast telomere biology, as it is required to nucleate both capping and extending complexes to the DNA ends (Pennock *et al.*, 2001). Genetic studies demonstrate that Cdc13 protects chromosomal termini along with Stn1– Ten1 and is required to extend the telomeric DNA in conjunction with the telomerase holoenzyme (Gilson and Geli, 2007; Shore and Bianchi, 2009). To determine whether Hsp82 influences Cdc13-mediated events, I helped to establish an in vitro system in which

Cdc13 alone could stimulate telomerase DNA extension activity, but an unextendable telomeric DNA structure formed in the co-presence of Stn1–Ten1 (DeZwaan *et al.*, 2009). Significantly, addition of Hsp82 dissociated the Cdc13–Stn1–Ten1–DNA complex but did not impede the ability of Cdc13 to stimulate telomerase. Hence, Hsp82 permitted a transition between the capping and extending assemblies without interfering with the positive telomerase regulatory events provided by Cdc13. Whether this disassembly activity explains why HSP82 is a high-copy suppressor of the cdc13-1 and stn1-157 mutations remains unknown. However, the ability of Hsp82 to promote DNA dissociation (i.e. Cdc13) and association (i.e. telomerase) of different telomere proteins might account for the extensive interaction network between Hsp82 and telomere proteins, as it would create a self-organizing telomere environment.

To illustrate, I expand upon the conceivable events that occur in late S phase when telomeres are directed to either extendable or unextendable states (Shore and Bianchi, 2009). Initial expectations posited that all telomeric DNA would be lengthened during the duplication of each chromosome (Shampay and Blackburn, 1988; Blackburn *et al.*, 1989). However, direct testing showed that individual telomere size is not increased in every cell cycle (Teixeira *et al.*, 2004). Furthermore, the extended telomeres displayed a sizeable variability in the amount of DNA added, as the interquartile range in extension length was 14–80 nucleotides. To account for the distribution in extension lengths, telomeres must be governed by a system that reacts continuously to regulatory signals and can control telomerase action before and after each added telomeric DNA repeat. To be able to respond to the incoming signals, I suspect that the HSP90 chaperone system maintains the telomere environment in a dynamic state (Figure 18). In this model, Hsp82

promotes the continual dissociation of the unextendable complex (Cdc13–Stn1–Ten1), which will reform unless the requisite components and signals for constructing the extendable structure are present. For example, assembly of the telomerase holoenzyme is restricted to S-phase because the essential Est1 subunit is expressed only at this point of the cell cycle (Osterhage *et al*., 2006). In addition, the actions of certain kinases (e.g. cyclin-dependent kinase 1 (Cdk1) and Tel1) appear to guide telomeres towards the requisite state by minimally modifying Cdc13. Cdk1-mediated phosphorylation likely disfavors Cdc13 interactions with Stn1 and fosters association with Est1 (Li *et al*., 2009). In addition, the Tel1 kinase is required for telomere DNA maintenance, and its potential phosphorylation targets include Cdc13 and the MRX complex subunit Xrs2 (Nakada *et al*., 2003; Tseng *et al*., 2007). However, the precise mechanistic contributions of Tel1 signaling are incompletely understood.

After nucleation of the modified telomerase holoenzyme at a telomere, Hsp82 and its co-chaperone Sba1 are likely to promote a cyclical telomerase action, as Hsp82 supports DNA binding by telomerase and Sba1 dissociates telomerase DNA complexes (Toogun *et al*., 2007; Toogun *et al*., 2008). How Hsp82 promotes binding while Sba1 facilitates release is not clear, though it might occur through a chaperone-mediated opening and closing of the DNA-binding cleft. Nevertheless, rapid cycling of telomerase on and off the telomeric DNA would be beneficial for effective extension of the chromosomal termini. Following sufficient telomeric DNA lengthening, Sba1-mediated dissociation of the telomerase complex and action by unidentified phosphatases (which would reverse the influence of the Cdk1 and Tel1 kinases), are likely to promote the reformation of the Cdc13–Stn1–Ten1 unextendable structure. Hence, by maintaining the

telomere components in a dynamic state, the Hsp82 chaperone machine would permit the system to transition efficiently between different operative states, which are guided by post-translational modifications and the presence of the required subunits; the chaperone-mediated interchange would also foster effective action for complexes that require movement (e.g. telomerase-mediated DNA extension).

In order to understand how complexes transition effectively, it is also necessary to grasp the functional role(s) of the individual components that come together to form these multi-subunit structures. In the case of the telomerase extending complex an essential component, Est1, remained uncharactierized for years due to an inability to purify the protein. The lack of functional Est1 information made understanding the mechanism and potential role(s) of the extending complex complicated. However, I was able to successfully and reproducibly purify Est1 and through my extensive biochemical characterization of Est1 in conjunction with previous EST protein data, the field is beginning to shed light on a model of EST protein function in telomerase extension. Based on the available data, I favor a model in which yKu70/80 initiates telomerase telomere association then, depending upon the available components, the enzyme will assimilate into an "EST" holoenzyme structure (Figure 19). The relative stability of the assembling complex will vary according to which components are present. For example, Est1 expression is restricted to S-phase and Est3 is reliant upon Est1 to associate with telomerase (Osterhage *et al*., 2006). Therefore, nucleation of the telomerase EST holoenzyme is only possible during this stage of the cell cycle, which likely limits the timing of telomeric DNA extension to S-phase (Marcand *et al*., 2000).

I suspect that the EST proteins cooperatively associate to both control telomerase

activity and to stabilize the telomere-bound structure. Such reliance would explain the co-dependence for telomere interactions by the EST proteins (Taggart and Zakian, 2003; Fisher *et al*., 2004; Chan *et al*., 2008). Hence, a disruption in any one of the holoenzyme subunits would result in declined telomere occupancy for all the components. Conversely, localization of any single subunit to a telomere should nucleate the entire holoenzyme. For instance, tethering either Est1, Est3 or Cdc13 near a DNA end, either at a natural telomere or a HO-induced double-strand break, is sufficient to nucleate an active telomerase complex (Evans and Lundblad, 1999; Hughes *et al*., 2000; Bianchi *et al*., 2004). In addition to the EST connections, genetic interactions between yKu70/80 and Est1 or Est2 have been reported (Evans and Lundblad, 2002; Stellwagen *et al*., 2003). Unfortunately, the only assay (ChIP) capable of detecting in vivo protein DNA interactions cannot differentiate between recruitment and stability effects, as it relies on a chemical crosslinker to capture the bound complexes. Thus, it is difficult to distinguish whether a factor delivers or steadies protein DNA assemblies in vivo.

Compelling points to consider yKu70/80 as the primary telomerase recruitment factor are the relative magnitude of yKu70/80's effect on Est2 telomere occupancy and the sufficiency of yKu70/80 to engage telomerase at a telomere in G1 (Fisher *et al*., 2004; Chan *et al*., 2008). If, however, yKu70/80 normally initiates loading of telomerase, then why isn't there a more significant telomere DNA maintenance phenotype (length is stably reduced ~2-fold) in *yku70*D yeast (Porter *et al*.,1996)? Perhaps the shared ability to bind telomeric DNA by yKu70/80, Est1, Cdc13 and telomerase circumvents a reliance on any single factor to engage telomerase at a telomere. Given the catastrophic physiological outcome of even a single critically shorten telomere (*i.e*., replicative senescence) it would

seem prudent that compensatory mechanisms exist to overcome deficiencies (Hemann *et al*., 2001). Since individual telomeres do not need to be lengthened in each cell cycle (Teixeira *et al*., 2004), a less efficient telomere recruitment route (*i.e*., Est1-mediated) might suffice in the absence of the primary pathway (*e.g.*, *yku70*D yeast). If Est1 isn't the main telomerase recruitment factor then why is Est1 so critical for telomerase DNA maintenance?

Biochemical work has provided insights into the direct regulatory contributions that Est1 makes to telomerase. Purified Est1 binds selectively to both telomeric DNA and the Tlc1 RNA bulged-stem loop (Vitra-Pearlman *et al*., 1996; DeZwaan and Freeman, 2009). the in vitro RNA binding specificity agrees with in vivo work (Livengood *et al*., 2002; Seto *et al*., 2002). However, my work found that neither nucleic acid interaction appears to be required to control the enzymatic activity of telomerase (DeZwaan and Freeman, 2009). Rather, Est1 associates directly with the Est2 reverse transcriptase to stimulate enzymatic activity. Comparably, human Est1A can directly bind to the human telomerase protein subunit in the absence of the RNA template (Redon *et al*., 2007). Hence, the main influence of Est1 on telomerase in vitro is to stimulate its DNA extension activity by directly contacting the reverse transcriptase protein.

In addition to Est1, Cdc13 has been shown to stimulate the DNA extension function of telomerase in vitro. (DeZwaan *et al*., 2009). While both Est1 and Cdc13 have innate abilities to modulate telomerase activity, at concentrations that likely reflect physiological levels neither protein displayed a significant effect (DeZwaan and Freeman, 2009). Notably, it was shown that Est1 and Cdc13 could jointly up-regulate telomerase activity at lower protein amounts and the cooperative Est1 Cdc13 effect was

dependent upon the 252/444 salt-bridge. Of note, the Est1-60 protein did not efficiently activate telomerase even at high concentrations (DeZwaan and Freeman, 2009). In conjunction with the available ChIP data, the telomere DNA maintenance phenotypes of the *est1-60* and *cdc13-2* yeast likely result from a defect in telomerase up-regulation rather than impaired telomere recruitment (Evans and Lundblad, 1999; Pennock *et al.*, 2001; Taggart *et al.*, 2002; Chan *et al*., 2008; DeZwaan and Freeman, 2009).

I suspect that the interaction between Est1 and Cdc13 also stabilizes an EST-holoenzyme to the telomeres, which would account for the observed minor decline in telomerase telomere occupancy in the *cdc13-2* yeast (Chan *et al.*, 2008). Though a lesser Est1 Cdc13 role in telomerase recruitment cannot be disregarded. However, since a more significant decrease in telomerase telomere occupancy can be observed without inducing an EST phenotype (*i.e*., *yku70*D yeast), I believe that the Est1 Cdc13 interaction primarily impacts regulation of telomerase DNA extension activity.

In conclusion, telomere protein biology serves as an excellent molecular paradigm to understand how the efficiency of a single system can impact homeostasis, as telomere dysfunction can lead to either cellular senescence or uncontrolled growth (*i.e*., cancer). Given the number of employed proteins with common binding specificities (Figure 1), a decline in telomere efficiency is highly plausible due to complex misassembly or impaired structure disassembly. The fundamental properties of molecular chaperones (abundant proteins with promiscuous but weak binding activities) would help proteins in the telomere system like Cdc13, Stn1, Ten1 or Est1 (low abundance, most likely having stronger binding activities) avoid these challenges by cultivating a self-organizing environment. In essence, the ability of the HSP90 chaperone network to foster telomere

protein dynamics parallels the more established molecular chaperone roles in protein folding in which a chaperone does not dictate the final folded structure (path direction) but rather helps the nascent chain (system) avoid off-pathway energy barriers that commonly occur in protein folding (multi-step) energy landscapes.

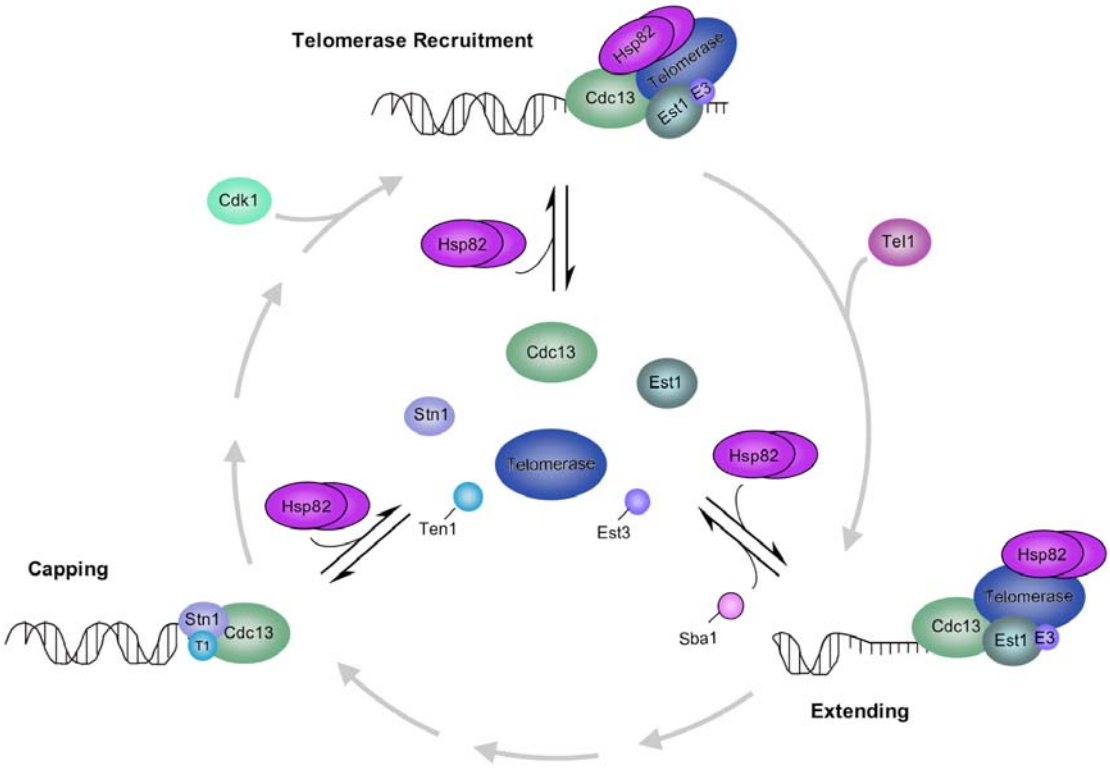

Figure 18. HSP90 maintains the telomere environment in a dynamic state that permits rapid and efficient reactions to incoming signals and components. In late S phase, telomeres are guided to either a telomerase extendable state or maintained in a protective unextendable form depending upon the length of the adjacent telomeric DNA (Shore and Bianchi, 2009). In order to transition to a required structure during the narrow time frame afforded in late S phase, telomere structures are likely continuously disassembled and reassembled into various complexes depending upon the available components (*e.g.*, Stn1–Ten1 vs. telomerase–Est1–Est3) and local signals (*e.g.*, Cdk1 and Tel1 kinases). In yeast, Hsp82 fosters both dissociation (protection components) and association (extension subunits) of telomere structures. In conjunction with the Sba1 co-chaperone that destabilizes the extension complex, telomeres are able to efficiently transition between the different functional states in order to properly maintain the chromosomal termini.

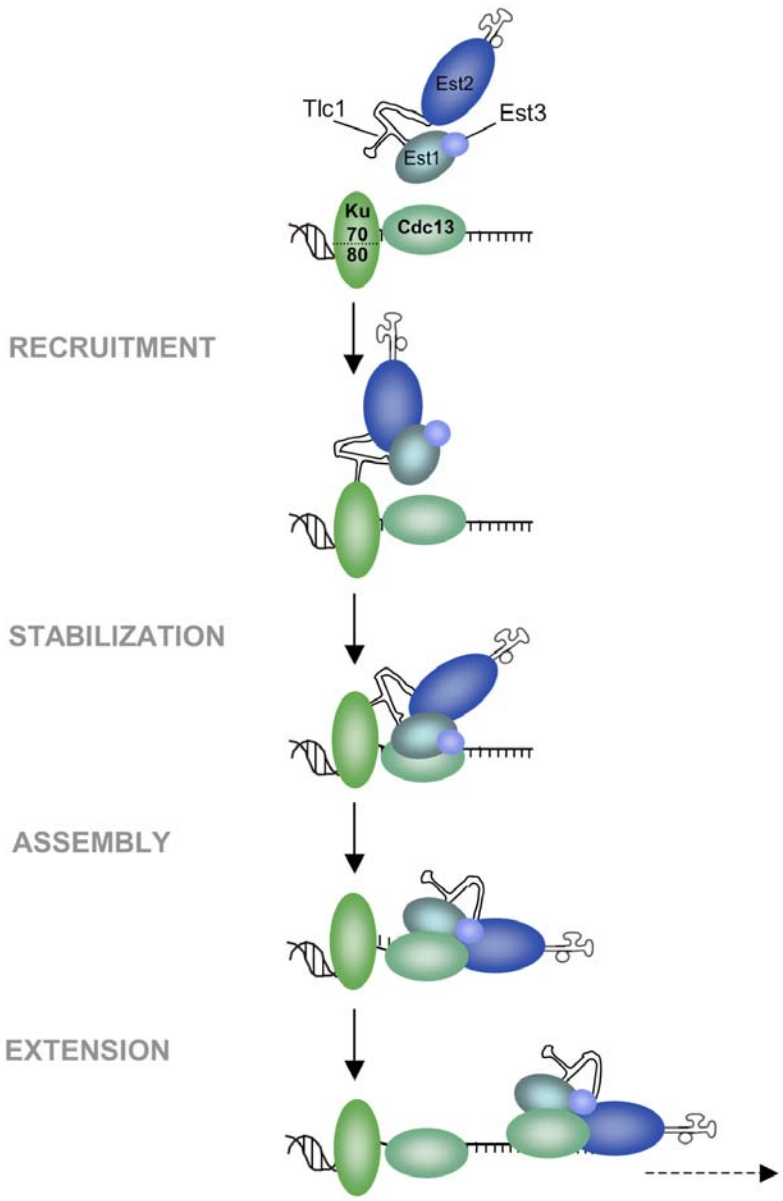

Figure 19. Est1 stimulation of telomerase enzymatic activity is critical for proper telomere DNA lengthening. Before telomere-association the telomerase holoenzyme assembles through an interaction between Est1 and the bulge-stem loop region of the TLC1 RNA. The Ku70/80 protein complex recruits telomerase to the telomere through the association with a different segment of the TLC1 RNA. At the telomere, Est1 interacts with Cdc13 via their salt-bridge and this association helps to stabilize the enzyme so it can fully assemble. Once assembled, Est1 and Cdc13 synergistically stimulate telomerase to extend the DNA.

CHAPTER 5

MATERIALS AND METHODS

Telomeres are specialized nucleoprotein complexes found at the ends of eukaryotic chromosomes that serve at least two functions: (1) DNA end protection and (2) linear DNA length maintenance (McClintock, 1941; Watson, 1972). The majority of Eukaryotes use an enzyme known as telomerase to sustain the telomeric end (Gilson and Geli, 2007). The core of telomerase is composed of a reverse transcriptase (Est2 in yeast) and an RNA template (Tlc1 in yeast) (Singer and Gottschling, 1994; Lingner *et al.*, 1997). The telomerase holoenzyme consists of two additional co-factors (Est1 and Est3 in yeast), however, their telomere maintenance function(s) in association with telomerase are not well understood. The G-rich single-stranded DNA of the telomere is also recognized and bound by a multitude of other proteins to form a variety of telomere-protein assemblies including chromosome extending, capping and replicating complexes (Smogorzewska and de Lange, 2004; Gilson and Geli, 2007). Significantly, the Hsp90 machinery has been shown to affect multiple telomere components, however, its potential roles with these complexes have remained unclear (Holt *et al*., 1999). The focus of this chapter will be to describe several methodologies designed to help understand the roles of various proteins involved in telomere maintenance. The specific goals of these assays were to characterize the telomerase co-factor Est1 as a means of understand its function with telomerase and also to explore the role(s) the Hsp90 molecular chaperone is performing with different telomere-proteins assemblies, including the capping complex and the telomerase holoenzyme.

Materials and Reagents

For the assays:

[1] Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma).

[2] DEAE fast flow sepharose (Pharmacia-LKB)

[3] ResourceQ resin (Pharmacia-LKB)

[4] MonoQ resin (Pharmacia-LKB)

[5] Superdex-200 size exclusion column (Pharmacia-LKB)

[6] Monoclonal Anti-HA-Agarose (Sigma)

[7] IgG Sepharose 6 Fast Flow (GE)

[8] Streptavidin Magnesphere Paramagnetic Particles (Promega)

[9] [α-^{32}P]GTP (MP Biomedical)

[10] [γ-^{32}P]ATP (MP Biomedical)

[11] [α-^{32}P] UTP (MP Biomedical)

[12] [35^{S}] Methionine (MP Biomedical)

[13] Chicken egg white lysozyme (USB)

[14] Micro-concentrator (Amico)

[15] Raffinose/Galactose (Difco)

[16] 10xAmino Acid drop-out mixture (Sigma)

[17] α-Factor

[18] Amino acids (His, Ura, Trp, Leu, Lys, Arg, Gln)

[19] dNTPs (Amersham Biosciences)

[20] ATP

[21] Creatine Phosphate/Creatine Kinase (Sigma)

[22] NaF, β-Glycerophosphate, Na_3VO_4 (Phosphotase Inhibitors)

[23] Talon Metal Affinity Resin (Clontech)

[24] Yeastar Yeast Genomic DNA isolation kit (Zymo Research)

[25] T'n'T Quick Coupled System (Promega)

[26] Ampicillin, Kanamycin, Streptomycin (USB)

[27] RNasin RNase Inhibitor (Promega)

[28] Polydeoxy(Inodsinate-Cytidylate) (dIdC)

[29] T7 Maxishortscript In Vitro Transcription Kit (Ambion)

[30] RNeasy Mini kit (Qiagen)

[31] Speed vacuum

[32] Hybridization oven (Bellco)

[33] Crosslinking machine (Spectro-Linker)

[34] α-Aminoadipate

Specific equipment needed:

[1] Fast performance liquid chromatography (FPLC) system (Pharmacia-LKB)

[2] Phosphoimager (Molecular Dynamics)

[3] Precision water baths of which the temperature can be regulated ± 0.1°C

[4] DNA Sequencing gel rig (Owl Separation Systems)

[5] Heated sand blocks

[6] Electromobility shift assay gel running apparatus

[7] 18 °C and 30 °C shaker for batch cell growth

[8] Replica Plating apparatus

1. Expression of Est1 in bacteria cells:

[1] Recombinant expression of wild type Est1 was best accomplished using the pET28-Est1 construct.

[2] For recombinant expression of Est1 we used Rosetta *Escherichia coli* cells (Novagen) transformed with the pET28-Est1 construct.

NOTE [1]: The Novagen Rosetta cells are designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli.* These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons.

[3] Luria-Bertani (LB) Media: dissolve 37.5 g LB media in dH_2O (total volume 1.5 L) then autoclave.

[4] Inoculate the transformant into a 500 mL LB flask (500 μL 35 mg/mL Kanamycin (Kan)) and allow to shake between 16-20 hours at 30 °C. (Overnight culture)

[5] Seed eight 1.5 L LB (plus Kan) flasks with the overnight culture to an optical $density_{595}$ (O.D.) of 0.1.

[6] Let the 1.5 L cultures shake at 18 °C until the O.D. reaches 0.3.

[7] Induce the cultures with 50 mM IPTG.

[8] Allow the cultures to shake at 18 °C for an additional 45 minutes.

[9] Clarify the cells (5,000 RPM, 7 minutes, 4 °C), keeping the remaining cultures on ice as it waits to be clarified.

[10] Resuspend the bacterial pellet on ice in 10-15 mL of 1x Talon Binding Buffer (4 °C) with 1x Protease inhibitor cocktail (PI = Protease Inhibitor cocktail made of Aprotinin, Leupeptin and Pepstatin) and PMSF (both at 1 mM).

[11] Transfer the resuspension to a 50 mL conical on ice, and add 0.5 μg/mL chicken egg white lysozyme to the cells and nutate for 5 minutes at 4 °C.

[12] Snap freeze the cells in a mixture of dry ice and methanol.

[13] Perform a series of "freeze/thaws" in which the pellet is initially snap frozen (Step 12) for ~10 minutes until completely solid, then the pellet is thawed at 37 °C until the pellet is just liquefied (~10 minutes). This cycle is repeated until there has been a total of four "freezes" and three "thaws", ending on a freeze before storage at -80 °C.

[14] 1x Talon Binding Buffer (50 mM KPO_4 pH 7.0, 300 mM NaCl, 10% Glycerol). Store at 4 °C.

2. Expression of Cdc13 in bacteria cells:

[1] Recombinant expression of wild type Cdc13 was best accomplished using the pET28-Cdc13 construct.

[2] For recombinant expression of Cdc13 we used streptomycin-dependent SmP *Escherichia coli* cells transformed with the pET28-Cdc13 construct (Siller *et al*., 2010).

NOTE [2]: The SmP cells were used since they slow the translational rate of proteins, allowing for more time for nascent proteins to fold properly.

[3] Luria-Bertani (LB) Media: dissolve 37.5 g LB media in dH_2O (total volume 1.5 L) then autoclave.

[4] Inoculate the transformant into a 500 mL LB flask (500 μL 35 mg/mL Kan; 500 μL 200 mg/mL Streptomycin) and allow to shake between 16-20 hours at 30 °C. (Overnight culture)

[5] Seed eight 1.5 L LB (plus Kan, no Streptomycin needed at this point) flasks with the overnight culture to an $O.D._{595}$ of 0.1.

[6] Let the 1.5 L cultures shake at 18 °C until the O.D. reaches 0.3.

[7] Induce the cultures with 100 mM IPTG.

[8] Allow the cultures to shake at 18 °C for an additional 4 hours.

[9] Clarify the cells (5,000 RPM, 7 minutes, 4 °C)

[10] Resuspend the bacterial pellet on ice in 10-15 mL of 1x Talon Binding Buffer (4 °C) with 1x PI and PMSF.

NOTE [3]: 1% NP-40 can be added to the 1x Talon Binding Buffer.

[11] Transfer the resuspension to a 50 mL conical on ice, and add 0.5 μg/mL chicken egg white lysozyme to the cells and nutate for 5 minutes at 4 °C.

[12] Snap freeze the cells in a mixture of dry ice and methanol.

[13] Perform a series of "freeze/thaws" in which the pellet is initially snap frozen (Step 12) for ~10 minutes until completely solid, then the pellet is thawed at 37 °C until the pellet is just liquefied (~10 minutes). This cycle is repeated until there has been a total of four "freezes" and three "thaws", ending on a freeze before storage at -80 °C.

[14] 1x Talon Binding Buffer (50 mM KPO_4 pH 7.0, 300 mM NaCl, 10% Glycerol). Store at 4 °C.

3. Expression of Stn1 or Ten1 in bacteria cells:

[1] Recombinant expression of wild type Stn1 or Ten1 was best accomplished using the pET-DUET-Stn1 or pET-DUET-Ten1 construct.

[2] For recombinant expression of Stn1/Ten1 we used Rosetta *Escherichia coli* cells (Novagen) transformed with the pET-DUET-Stn1 or the pET-DUET-Ten1 construct.

[3] Luria-Bertani (LB) Media: dissolve 37.5 g LB media in dH_2O (total volume 1.5 L) then autoclave.

[4] Inoculate the transformant into a 500 mL LB flask (500 μL 100 mg/mL Ampicilin (Amp)) and allow to shake between 16-20 hours at 30 °C. (Overnight culture)

[5] Seed eight 1.5 L LB (plus Amp) flasks with the overnight culture to an optical $density_{595}$ (O.D.) of 0.1.

[6] Let the 1.5 L cultures of Stn1 shake at 30 °C until the O.D. reaches 1.0 (can also be done at 18°C) and let the 1.5 L cultures of Ten1 shake at 18°C until the O.D. reached 1.0.

[7] Induce the cultures with 50 mM IPTG.

[8] Allow the Stn1 cultures to shake at 30°C for 60 minutes (if at 18 °C, for an additional 3hours). Allow the Ten1 cultures to shake at 18°C for 4 hours.

[9] Clarify the cells (5,000 RPM, 7 minutes, 4 °C).

[10] Resuspend the bacterial pellet on ice in 10-15 mL of 1x Talon Binding Buffer (4 °C) with 1x Protease inhibitor cocktail (PI = Protease Inhibitor cocktail made of Aprotinin, Leupeptin and Pepstatin) and PMSF (both at 1 mM). Ten1 should be brought up in 1x Talon Binding Buffer + 1% NP-40.

[11] Transfer the resuspension to a 50 mL conical on ice, and add 0.5 μg/mL chicken egg white lysozyme to the cells and nutate for 5 minutes at 4 °C.

[12] Snap freeze the cells in a mixture of dry ice and methanol.

[13] Perform a series of “freeze/thaws” in which the pellet is initially snap frozen (Step 12) for ~10 minutes until completely solid, then the pellet is thawed at 37 °C until the

pellet is just liquefied (~10 minutes). This cycle is repeated until there has been a total of four “freezes” and three “thaws”, ending on a freeze before storage at -80 °C.

[14] 1x Talon Binding Buffer (50 mM KPO_4 pH 7.0, 300 mM NaCl, 10% Glycerol). Store at 4 °C.

4. Yeast (*Saccharomyces cerevisiae*) Growth:

[1] Plate the *Saccharomyces cerevisiae* yeast on Yeast Extract, Peptone, Dextrose (YPD) plates and incubate for 1-2 days at 30 °C.

[2] YPD Media: dissolve 50 g YPD media in dH_2O (total volume 1 L) then autoclave.

[3] Inoculate a 500 mL YPD overnight culture and shake between 18-36 hours at 30 °C.

[4] Seed twelve 1 L YPD flasks with the overnight culture to an $O.D._{595}$ of 0.25.

[5] Allow the cultures to shake at 30 °C until the $O.D._{595}$ reaches 1.0

[6] Clarify the cells (3,000 RPM, 7 minutes, 4 °C)

NOTE [4]: Resuspension Buffer is dependent on the type of yeast grown.

[7] For yeast used to make telomerase extracts: Resuspend the cell pellet on ice in 25 mL of Lysis Buffer (4 °C).

[8] For HA-epitope tagged yeast: Resuspend the cell pellet on ice in 15 mL of Tel1 Buffer (4 °C).

[9] For Tap-epitope tagged yeast: Resuspend the cell pellet on ice in 15 mL YEB Buffer (4 °C).

[10] Clarify the cells (2,500 RPM, 5 minutes, 4 °C)

[11] Snap freeze the cells in a mixture of dry ice and methanol and store at -80 °C.

[12] Lysis Buffer: 20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 500 mM NaoAc pH 5.0, 10% glycerol, 0.1% Triton x-100 and 0.2% NP-40. Store at 4 °C.

[13] Tel1 Buffer: 10 mM Tris pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.2% Tween-20, 1 mM DTT, 1x Phosphotase Inhibitors, 1 mM PMSF. Store at 4 °C.

[14] YEB Buffer: 245 mM KCl, 1 mM EDTA, 1 mM EGTA, 100 mM HEPES pH 6.9 and 2.5 mM DTT. Store at 4 °C.

NOTE [5]: Add the EGTA and DTT to the YEB Buffer immediately before usage.

5. Est2 In Vitro Transcription and Translation:

[1] Use the Quick Coupled Transcription and Translation (T'n'T) reaction kit from Promega.

[2] Thaw the T'n'T on ice.

[3] In a 1.5 mL snap cap tube on ice, add 40 μL of T'n'T Quick Master Mix.

[4] To this add 2 μL [35^S] Methionine, 1 μg T7-ProA-Est2, 1 μL T7 T'n'T PCR Enhancer and 3 μL dH_20 (Friedman and Cech 1999).

NOTE [6]: The T7 T'n'T PCR Enhancer must be added, otherwise the reaction does not work. 1 μg of the Tlc1 Mini-T plasmid can be added to incorporate Tlc1 into Est2 (Zappulla *et al*., 2005).

[5] Incubate the reaction for 90 minutes at 30 °C.

[6] Place on ice or freeze at -20°C until usage.

6. Est1 Purification:

[1] 1x Talon Binding Buffer (50 mM KPO_4 pH 7.0, 300 mM NaCl, 10% Glycerol)

[2] Talon Metal Affinity Resin (Clontech)

[3] Imidazole

[4] 20 mL Poly-prep chromatography column (Biorad)

[5] FPLC

[6] MonoQ resin

[7] TenG Buffer (20 mM Tris pH 6.9, 0.1mM EDTA, 10% Glycerol)

[8] TenG1 Buffer (20 mM Tris pH 6.9, 0.1mM EDTA, 10% Glycerol, 1 M NaCl)

[9] TMG-30 pH 7.0 Buffer (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 30 mM NaoAc pH 7.0)

[10] Micro-concentrator (Amico)

7. Cdc13 Purification:

[1] 1x Talon Binding Buffer (50 mM KPO_4 pH 7.0, 300 mM NaCl, 10% Glycerol)

[2] Talon Metal Affinity Resin (Clontech)

[3] Imidazole

[4] FPLC

[5] MonoQ resin

[6] Superdex Size Exclusion Column

[7] TenG Buffer (20 mM Tris pH 6.9, 0.1mM EDTA, 10% Glycerol)

[8] TenG1 Buffer (20 mM Tris pH 6.9, 0.1mM EDTA, 10% Glycerol, 1 M NaCl)

[9] TMG-30 pH 7.0 Buffer (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 30 mM NaoAc pH 7.0)

[10] Micro-concentrator (Amico)

8. Ten1 Purification:

[1] 1x Talon Binding Buffer + 1% NP-40 (50 mM KPO_4 pH 7.0, 300 mM NaCl, 10% Glycerol, 1% NP-40)

[2] Talon Metal Affinity Resin (Clontech)

[3] Imidazole

[4] FPLC

[5] MonoQ resin

[6] Superdex Size Exclusion Column

[7] TenG Buffer + 1% NP-40 (20 mM Tris pH 6.9, 0.1mM EDTA, 10% Glycerol, 1% NP-40).

[9] TMG-30 pH 7.0 Buffer (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 30 mM NaoAc pH 7.0)

[10] Micro-concentrator (Amico)

9. Stn1 Purification:

[1] Inclusion Body Wash Buffer (100 mM Tris pH 7.5, 100 mM NaCl)

[2] 8 M Urea

[3] Talon Metal Affinity Resin (Clontech)

[4] Denaturing Buffer (50 mM Tris pH 8.0, 100 mM NaCl, 10 mM DTE, 8 M Urea)

[5] Refolding Buffer (100 mM Tris pH 7.5, 300 mM NaCl, 50 mM Arginine, 50 mM Glutamine)

[6] 20 mL Poly-prep chromatography column (Biorad)

[7] Imidazole

[8] Elution Buffer (40 mM NaP pH 7.8, 300 mM NaCl, 500 mM Imidazole, 10 mM DTE, 10 mM DTT, 1% NP-40)

10. Telomerase Extract Preparation:

[1] Lysis Buffer (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 0.2% NP-40, 500 mM NaoAc pH 5.0) Add RNasin RNA inhibitors immediately before usage.

[2] Coffee grinder

[3] TMG-500 pH 5.0 (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 500 mM NaoAc pH 5.0)

[4] TMG-1000 pH 5.0 (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 1 M NaoAc pH 5.0)

[5] FPLC

[6] DEAE Resin

[7] MonoQ resin

[8] 1 mL and 3 mL syringe

[9] TMG-30 pH 7.0 Buffer (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 30 mM NaoAc pH 7.0)

[10] 80% Glycerol

[11] Micro-concentrator (Amico)

11. DNA-Binding EMSA:

[1] 20x GTG Buffer (1.78 M Tris, 0.57 M Taurine, 10 mM EDTA)

NOTE [7]: 20x GTG (Glycerol Tolerant Gel) is used to prevent gel distortions associated with samples that contain glycerol. Taurine does not react with glycerol while sodium borate does and will cause distortion.

[2] EMSA electrophoresis apparatus

[3] [γ-^{32}P] ATP (MP Biomedical)

[4] dIdC (Amersham Biosciences)

[5] TMG-30 pH 7.0 Buffer (20 mM Tris pH 8.0, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 0.1% Triton x-100, 30 mM NaoAc pH 7.0)

[6] Native Loading Dye

12. RNA-Binding EMSA:

[1] 20x GTG Buffer (1.78 M Tris, 0.57 M Taurine, 10 mM EDTA)

[2] EMSA electrophoresis apparatus

[3] RNA-Binding Buffer (10 mM HEPES pH 7.8, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 3% Ficoll) 1 mM DTT, Heparin (1mg/mL) and RNasin RNase inhibitor are added immediately before usage (Vitra-Pearlman *et al*., 1996).

[4] T7 Maxishortscript In Vitro Transcription kit (Ambion)

[5] RNeasy Mini kit (Qiagen)

[6] Native Loading Dye

13. Telomerase Extension Assay:

[1] 10 x Telomerase Buffer (500 mM Tris pH 8.0, 10 mM $MgCl_2$, 10 mM Spermidine, 10 mM DTT, 500 μM dTTP)

[2] Streptavidin Magnesphere Paramagnetic Particles (Promega)

[3] [α-^{32}P]GTP (MP Biomedical)

[4] EcoRI Buffer (50 mM NaCl, 100 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.025% Triton x-100)

[5] Magnetic rack for 1.5 mL tubes

[6] DNA sequencing electrophoresis apparatus (Owl)

[7] Urea

[8] Formamide Buffer + NaOH (80% Formamide, 10 mM EDTA, 1 mg/mL xylene cyanol, 1 mg/mL Bromophenol blue, 30mM NaOH)

[9] Heated sand block (95 °C)

[10] Speed Vacuum

14. Co-Immunoprecipitation:

[1] Quick Coupled T'n'T Master mix (Promega)

[2] IgG Sepharose 6 Fast Flow

[3] TMG Buffer (10 mM Tris pH 8.0, 1 mM $MgCl_2$, 10% Glycerol)

[4] Heated sand block

15. Telomere Southern:

[1]Yeastar yeast genomic DNA isolation kit (Zymo Research)

[2] Denaturing Buffer (87.6 g NaCl, 20 g NaOH up to 1 L dH_20)

[3] Neutralization Buffer (1.5 M NaCl, 1 M Tris pH 8.0)

[4] 20x SSC (175.3 g NaCl, 88.2 g sodium citrate, pH to 7.0, adjust to 1 L with dH_20)

[5] Ekono Buffer (RPI)

[6] Hybridization Oven

[7] Crosslinker Machine (Spectro-Linker)

[8] dNTPs

[9] GTP

[10] [α-^{32}P]GTP (MP Biomedical)

[11] [γ-^{32}P]ATP (MP Biomedical)

[12] Nitrocellulose membrane (Amersham)

[13] PCR Machine (Bio-Rad Thermocycler)

16. Exo-T Nuclease Assay:

[1] dIdC

[2] Formamide Buffer + NaOH (80% Formamide, 10 mM EDTA, 1 mg/mL xylene cyanol, 1 mg/mL Bromophenol blue, 30mM NaOH)

[3] [γ-^{32}P]ATP (MP Biomedical)

[4] DNA sequencing electrophoresis apparatus

[5] Urea

17. Generating mutant Tlc1 yeast extracts:

[1] 10 x Amino Acid drop out mix lacking Tryptophan

[2] Yeast Nitrogen base without amino acids (Simple Media)

[3] Dextrose

[4] α-Aminoadipate

[5] Replica plating apparatus

Methods

1. Est1 Protein Purification:

[1] Thaw the Est1 pellet at 37 °C (H_20 bath) for ~ 10 minutes or until it is completely liquefied with no ice chunks, then immediately put it on ice and add 1x PI/PMSF.

[2] Sonicate the sample on ice using a large tip at 50% duty on a 7 level output for 20 seconds continuously.

NOTE [8]: Repeat the pulse until the consistency is close to that of H_20. The sample should be chilled between pulses so that it does not overheat.

[3] Clarify the sample (17,000 RPM, 20 minutes, 4 °C)

[4] While clarifying, pre-wash the Talon metal affinity resin by aliquoting ~5mL Talon bead slurry into a 50 mL conical, clarify (2,500 RPM, 2 minutes, 4 °C), dump the supernatant (S/N).

[5] Add 20 mL of 1 x Talon Binding Buffer (1xTBB) to resuspend the beads (Wash #1), clarify as before and dump S/N. Repeat this wash step one additional time and leave the beads on ice until the sample is ready to be added.

[6] Transfer the clarified Est1 S/N to the 50 mL conical that contains ~5 mL of pre-washed Talon Affinity Beads, add 1x PI/PMSF.

[7] Nutate the sample/beads for 1 hour at 4 °C. Clarify the sample (2,500 RPM, 2 minutes, 4 °C), dump S/N.

[8] Resuspend the beads in 20 mL 1xTBB + PI/PMSF and re-clarify as above, repeat this wash step two additional times always making sure that PI/PMSF are added.

[9] The sample/beads should now undergo a series of Imidazole washes via a gravity flow column (20 mL BIO-RAD Poly-Prep Chromatography Column) using increasing concentrations of Imidazole to wash away *E. coli* contaminating proteins:

Wash A = Add 10 mL of a 10 mM Imidazole/1xTBB (PI/PMSF) solution to the beads and then add the solution to a gravity flow column at 4 °C. Allow the entire 10 mL to flow through before adding the next wash, discard flow through.

Wash B = Add 10 mL of a 20 mM Imidazole/1xTBB (PI/PMSF) solution slowly along the side of the column wall to avoid disrupting the bead bed. Allow the solution to completely flow through, discard flow through.

Wash C = Add 10 mL of a 40 mM Imidazole/1xTBB (PI/PMSF) solution as before

Elution = Add 7.5 mL of 100 mM Imidazole/1xTBB (PI/PMSF) and collect the flow through in a new 50 mL conical.

[10] Equilibrate the 7.5 mL elution with 22.5 mL of chilled TenG Buffer.

[11] Load this 30 mL sample onto a 1 mL Resource Q ion-exchange column at a 1 mL/min flow rate.

[12] To remove additional *E. coli* contaminants, wash the column for ~5 minutes in 450 mM TenG.

[13] Collect 1 mL fractions right when you set the gradient of 1000 mM TenG for 3 minutes.

[14] Concentrate (3,700 RPM, 4°C) the fractions in an Amico-microconcentrator (Add PI/PMSF prior to concentration) until the volume is ~100 μL.

[15] Equilibrate with 2 mL of chilled TMG-30 pH 7.0 buffer (PI/PMSF) and re-concentrate as above until the volume is 100 μL.

[16] Transfer the solution to a 1.5 mL tube on ice.

NOTE[9]: The protein must be used within 24 hours of purification, as it is extremely unstable. For the best result use Est1 immediately after purification.

2. Cdc13 Protein Purification:

[1] Thaw the Cdc13 pellet at 37 °C (H_20 bath) for ~ 10 minutes or until it is completely liquefied with no ice chunks, then immediately put it on ice and add 1x PI/PMSF

[2] Sonicate the sample on ice using a large tip at 50% duty on a 7 level output for 20 seconds continuously.

NOTE [10]: Repeat the pulse until the consistency is close to that of H_20. The sample should be chilled between pulses so that it does not overheat.

[3] Clarify the sample (17,000 RPM, 20 minutes, 4 °C)

[4] While clarifying, pre-wash the Talon metal affinity resin by aliquoting ~5mL Talon bead slurry into a 50mL conical, clarify (2,500 RPM, 2 minutes, 4 °C), dump the supernatant.

[5] Add 20 mL of 1 x Talon Binding Buffer (1xTBB) to resuspend the beads (Wash #1), clarify as before and dump the supernatant. Repeat this wash step one additional time and leave the beads on ice until the sample is ready to be added.

[6] Transfer the clarified Cdc13 supernatant to the 50 mL conical that contains ~5 mL of pre-washed Talon Affinity Beads, add 1x PI/PMSF.

[7] Nutate the sample/beads for 35 minutes at 4 °C. Clarify the sample (2,500 RPM, 2 minutes, 4 °C), dump the supernatant.

[8] Resuspend the beads in 20 mL 1xTBB + PI/PMSF and re-clarify as above, repeat this wash step two additional times always making sure that PI/PMSF are added

[9] Wash in 1 M NaCl 1xTBB, nutating for 10 minutes at 4 °C.

(1xTBB is already 300mM NaCl, so 7 mL 5M NaCl into 43 mL 1xTBB)

[10] Elute with 10 mL elution buffer (5 mL 1xTBB, 3 mL dH_20, 1 mL 1 M Imidazole and 1 mL 80% Glyercol. Add 5mL of this mixture initially to the beads and nutate at 4 °C for 15 minutes, clarify (2,500 RPM, 2 minutes, 4 °C) and save the supernatant on ice.

[11] Add the final 5 mL of the Imidazole mixture to the beads and nutate at 4°C for 10 minutes, clarify as before and add this to the saved the supernatant.

[12] Re-clarify (2,500 RPM, 2 minutes, 4 °C) the supernatant to remove any residual Talon resin.

[13] Take the 10 mL elution and add 30 mL TenG Buffer to equilibrate the salt.

[14] Load the 40 mL sample onto a ResourceQ column (2 mL/min) and allow the UV to baseline.

[15] Wash with TenG + 100 mM NaCl to remove additional *E. coli* contaminants and allow UV to baseline

[16] To elute the protein:

Full length Cdc13 = 100-300 mM NaCl gradient for 6 minutes (flow rate: 2 mL/min, collect 0.75 mL fractions)

DNA-Binding Domain of Cdc13 = 100-500 mM NaCl gradient for 6 minutes (flow rate: 2 mL/min, collect 0.75 mL fractions).

[17] Run out 10 μL of each fraction (5 μL 2xSB) on a 12% SDS-PAGE (240 V for 40 minutes). Concentrate the relevant fractions to ~200 μL in an Amicon microconcentrator (3,700 RPM, 4 °C for as long as it takes)

[18] Inject the sample into a Superdex sizing column equilibrated in TMG-30 pH 7.0 (flow rate: 0.25 mL/min) collect 0.6 mL fractions

[19] Run out 10 μL of each fraction (5 μL 2xSB) on a 12% SDS-PAGE (240 V for 40 minutes). Concentrate the relevant fractions to ~100 μL in an Amicon microconcentrator (3,700 RPM, 4 °C for as long as it takes), and store the sample at -20 °C.

3. Ten1 Purification:

[1] Thaw the Ten1 pellet at 37 °C (H_20 bath) for ~ 10 minutes or until it is completely liquefied with no ice chunks, then immediately put it on ice and add 1x PI/PMSF.

[2] Sonicate the sample on ice using a large tip at 50% duty on a 7 level output for 20 seconds continuously.

[3] Clarify the sample (17,000 RPM, 20 minutes, 4 °C).

[4] While clarifying, pre-wash the Talon metal affinity resin by aliquoting ~5mL Talon bead slurry into a 50 mL conical, clarify (2,500 RPM, 2 minutes, 4 °C), dump the supernatant (S/N).

[5] Add 20 mL of 1 x Talon Binding Buffer (1xTBB) + 1% NP-40 to resuspend the beads (Wash #1), clarify as before and dump S/N. Repeat this wash step one additional time and leave the beads on ice until the sample is ready to be added.

[6] Transfer the clarified Ten1 S/N to the 50 mL conical that contains ~5 mL of pre-washed Talon Affinity Beads, add 1x PI/PMSF.

[7] Nutate the sample/beads for 40 minutes at 4 °C. Clarify the sample (2,500 RPM, 2 minutes, 4 °C), dump S/N.

[8] Resuspend the beads in 20 mL 1xTBB + 1% NP-40 + PI/PMSF and re-clarify as above, repeat this wash step four additional times always making sure that PI/PMSF are added.

[9] Elute with 10 mL elution buffer (5 mL 1xTBB + 1% NP-40, 2 mL dH_20, 1 mL 1 M Imidazole and 1 mL 80% Glyercol. Add 5 mL of this mixture initially to the beads and nutate at 4 °C for 15 minutes, clarify (2,500 RPM, 2 minutes, 4 °C) and save the supernatant on ice.

[10] Add the final 5 mL of the Imidazole mixture to the beads and nutate at 4°C for 10 minutes, clarify as before and add this to the saved the supernatant.

[11] Re-clarify (2,500 RPM, 2 minutes, 4 °C) the supernatant to remove any residual Talon resin.

[12] Take the 10 mL elution and add 30 mL TenG + 1% NP-40 Buffer to equilibrate the salt.

[14] Load the 40 mL sample onto a ResourceQ column (2 mL/min)

[15] Immediately start collecting the flow through as one large fraction. (Ten1 will flow through the MonoQ, while the other contaminates will stick to the resin)

[16] Concentrate the large fraction in an Amicon microconcentrator (3,700 RPM, 4 °C for as long as it takes) to a volume of ~1 mL.

[17] Inject the sample into a Superdex sizing column equilibrated in TMG-30 pH 7.0 (flow rate: 0.25 mL/min) collect 0.6 mL fractions.

[18] Run out 10 μL of each fraction (5 μL 2xSB) on a 12% SDS-PAGE (240 V for 40 minutes). Concentrate the relevant fractions to ~100 μL in an Amicon microconcentrator (3,700 RPM, 4 °C for as long as it takes), and store the sample at -20 °C.

4. Stn1 Purification:

[1] Thaw the Stn1 pellet at 37 °C (H_20 bath) for ~ 10 minutes or until it is completely liquefied with no ice chunks, then immediately put it on ice and add 1x PI/PMSF.

[2] Sonicate the sample on ice using a large tip at 50% duty on a 7 level output for 20 seconds continuously.

[3] Clarify the sample (17,000 RPM, 20 minutes, 4 °C), discard the S/N and keep the pellet. (The pellet can be stored at -20°C at this stage for future usage).

[4] Thaw the Stn1 pellet at 30 °C (H_20 bath) for ~5 minutes and place immediately on ice, add 1xPI/PMSF.

[5] Resuspend the pellet in 15 mL (~5 mL/ 1 g) chilled Inclusion Body Wash Buffer, transfer the resuspension to a 50 mL conical tube on ice.

[6] Add an additional 15 mL of Inclusion Body Wash Buffer supplemented with 5% Triton x-100. Make sure that the pellet has been completely resuspended with no chunks.

[7] Nutate the sample for 25 minutes at 4 °C.

[8] Clarify the sample (17,000 RPM, 20 minutes, 4 °C), discard the S/N and keep the pellet. Rinse the pellet with 10 mL Inclusion Body Wash Buffer.

[9] Dissolve the pellet in 15 mL chilled 8 M Urea, vortexing vigorously until the pellet is completely dissolved (resuspended).

[10] Clarify the sample (17,000 RPM, 20 minutes, 4 °C).

[11] While clarifying, pre-wash the Talon metal affinity resin by aliquoting ~1mL Talon bead slurry into a 1.5 mL tube, clarify (3,000 RPM, 1 minutes, 4 °C), dump the supernatant (S/N).

[12] Add 1 mL of 8 M Urea to resuspend the beads (Wash #1), clarify as before and dump S/N. Repeat this wash step three additional time and leave the beads on ice until the sample is ready to be added.

[13] Transfer the clarified Stn1 S/N to a 50 mL conical on ice. Add the ~1 mL of pre-washed Talon Affinity Beads, add 1 mM DTE.

[14] Nutate the sample/beads for 30 minutes at 4 °C.

[15] Apply the mixture to a gravity flow column (20 mL BIO-RAD Poly-Prep Chromatography Column) at room temperature and allow the liquid to flow through completely.

[16] Apply 5 column volumes (CV) of Denaturing Buffer to the beads and allow the liquid to completely flow through.

[17] Apply 10 CV of Refolding Buffer to the column and allow the liquid to completely flow through, then cap the column and incubate on ice for 1 hour.

[18] With the column still capped, apply 200 μL of Elution Buffer to the beads, mixing up and down and then transferring to a 1.5 mL tube.

[19] Nutate the beads in the Elution Buffer for 10 minutes at 4 °C.

[20] Clarify the sample (3,000 RPM, 1 minute, 4 °C), transfer the S/N to a fresh 1.5 mL tube and re-clarify to remove any residual beads. Transfer the S/N to a fresh 1.5 mL tube on ice and Stn1 is ready for usage.

5. Telomerase extract preparation:

[1] Put the ~5 mL frozen yeast pellet on dry ice until you are ready to pulverize it.

[2] Chill 20 mL of Lysis Buffer on ice and add 1 mM PMSF, 1 mM DTT, 1 mM PI and 600 units of RNasin RNase inhibitor.

[3] Pre-chill a coffee grinder to 4 °C, add a small piece of dry ice to the coffee grinder and pulverize the ice to pre-chill the inside of the grinder.

[4] Add the frozen yeast pellet and an equal volume of dry ice to the coffee grinder and pulverize the sample until it is a consistent fine powder.

[5] Add the powder slowly to the Lysis Buffer, and swirl to generate bubbling from the dry ice.

NOTE[11]: There must be enough dry ice at this step, if the sample is “liquidy” once added to the Lysis Buffer due to lack of enough dry ice, the sample will not be good.

[6] Cap the tube loosely to avoid an explosion due to the dry ice inside. Nutate the sample at 4 °C until the sample has become completely liquefied.

[7] Clarify (17,000 RPM, 20 minutes, 4 °C), then pour the supernatant into a fresh 50 mL conical on ice that has a kimwipe placed over the opening. This will remove any residual lipids and other unpelleted contaminants.

[8] Add the telomerase extract supernatant to DEAE fast flow sepharose (Flow rate 2 mL/min). Allow the column to wash until the UV to baseline.

[9] Elute the protein: 0-100% B gradient over 20 minutes (flow rate: 2 mL/min, collect 2 mL fractions).

NOTE[12]: This will give a single broad peak, and you only want to pool the first half of the peak stopping at the climax. The majority of the telomerase activity is in the first half of the peak.

[10] Pool the relevant fractions and add chilled TMG-30 pH 7.0 Buffer to the sample in a ratio of 2:1 (e.g. 11 mL fractions to 22 mL TMG-30 pH 7.0 Buffer).

[11] Apply the diluted sample to MonoQ resin (Flow rate: 1 mL/min). Set your timer to catch the sample when there is less than 0.5 mL left to be injected over the column so that you can catch the “salt bump”.

NOTE[13]: As buffer begin to flow over the MonoQ resin, once the entire sample has been loaded onto the column, this creates a “salt bump” that dislodges the majority of the telomerase activity.

[12] Immediately start collecting fractions (0.5 mL fractions) as the telomerase activity will come off quickly(usually fractions 2-6).

[13] Concentrate the relevant fractions in an Amico-microconcentrator (3,700 RPM, 4 °C) until the sample is ~100 μL. Dilute the sample with 2 mL TMG-30 pH 7.0 Buffer and re-concentrate to ~100 μL.

[14] Add the sample to ~100 μL of pre-chilled 80% Glycerol, mix thoroughly, and store at -20 °C.

6. DNA-Binding Assay:

[1] Cast a 6% Acrylamide gel using 20 x GTG as the buffer (usually takes 1 hour to set). Place the gel upright in an EMSA electrophoresis apparatus and add 1 L of 1xGTG Buffer.

[2] To generate a hybrid telomere template:

PNK-end label the longer of the two primers with [γ-^{32}P]ATP. Clean the PNK-labeled sample by passing it through a mini-spin column (5,000 RPM, 5 minutes). Add equal amounts of the [γ-^{32}P]ATP –labeled primer to the shorter unlabeled primer in a 1.5 mL tube, mix (Total volume no more than 20 μL) , then boil in a 95 °C heated sand block for 3 minutes. Take the entire sand block off of the heat and place it directly on the tabletop and allow the sand block (with the hybrid template inside) to cool to room temperature. Once at room temperature, pulse the sample (2,500 RPM) to remove the liquid that has condensed on the lid of the tube. The hybrid is ready to use, or can be stored at -20 °C.

[3] Reactions are performed in 25 μL volumes. The protein of interest was added to TMG-30 pH 7.0 buffer along with 20 ng/μL dIdC, 0.2 μg/μL BSA and 250 pM DNA probe.

[4] Incubate the reaction at room temperature for 20 minutes.

[5] Add 1 μL of Native loading dye to each reaction and load onto the gel. Run the gel at 150 V for 120 minutes. Dry and expose the gel.

7. RNA-Binding Assay:

[1] Cast a 6% Acrylamide gel using 20 x GTG as the buffer (usually takes 1 hour to set). Place the gel upright in an EMSA gel rig and add 1 L of 1xGTG Buffer.

[2] The RNA transcripts were synthesized using the T7 Maxishortscript in vitro transcription kit (Ambion) in the presence of [α-^{32}P] UTP and purified using RNeasy mini kit (Qiagen).

[3] Transcripts were diluted to 32 nM, heat denatured (5 minutes at 95 °C) and snapped cooled before usage.

[4] The 10 μL reactions including the protein of interest were performed in RNA-Binding Buffer incubated on ice for 20 minutes (Vitra-Pearlman *et al.*, 1996).

[5] Add 1 μL of Native loading dye to each reaction and load onto the gel. Run the gel at 150 V for 120 minutes. Dry and expose the gel.

8. Telomerase Extension Assay:

NOTE[14]: Before performing an experiment you need to determine the amount of telomerase extract to use per reaction. This can be done by making a dilution series of 1 μL, 2 μL and 4 μL of a 1:100, 1:10 dilution and 1 μL, 2 μL and 4 μL straight. Perform these extensions on a 7-base overhang template and observe which dilution gives the preferred extension/intensity.

[1] To make the biotinilyated overhang template (200 μL Final Volume):

Mix 20 μL of 3'-Biotin modified telomerase oligonucleotide (100 μM), 20 μL of the specific overhang template oligonucleotide (100 μM), 10 μL 10x Annealing Buffer (100 mM Tris pH 7.3, 500 mM NaCl) and 50 μL dH_20. Boil for 3 minutes at 95 °C in a heated sand block. Then take the entire sand block off the heat and allow to cool to room temperature on the bench top. While cooling, pipette out 2 mL of Streptavidin Magnesphere Paramagnetic Particles (Promega) into a tube placed in a magnetic rack.

Pippette off the liquid and wash twice with 0.25 x SSC (1 mL per wash). Once the annealed overhang template has cooled to room temperature, add it to the paramagnetic particles, and allow to nutate at room temperature for at least 30 minutes. Then wash twice in TMG30 pH 7.0 Buffer (1 mL per wash) and bring to a final volume of 200 μL in TMG30 pH 7.0 Buffer and store at 4 °C.

[2] Aliquot dilutions of your protein into labeled 1.5 mL tubes so that the final volume is 5 μL (bring up to 5 μL in dH_20 or TMG-30 ph 7.0 buffer).

[3] Make a master mix sufficient for a 50 μL reaction volume per tube (5 μL 10x Telomerase Buffer, 1 μL [α-^{32}P] GTP, 1 μL overhang template, up to 50 μL dH_20). Pipette the Master mix into the tubes containing the protein of interest.

[4] Incubate at 30 °C for 30 minutes. Then place the tubes on a magnetic rack and allow the paramagnetic beads to collect against the side of the tube, pipetting off the supernatant into the radioactive trash.

[5] Wash the paramagnetic beads twice (200 μL) with EcoRI Buffer and discard supernatant leaving only the beads. Add 50 μL of EcoRI Digestion mix to the beads (1 μL EcoRI, 0.5 μL BSA (10 mg/mL) and 48.5 μL EcoRI Buffer per reaction).

[6] Incubate at 37 °C for 1 hour, then place the tubes back on the magnetic rack and allow the beads to collect on the side of the tube.

[7] Remove the 50 μL reaction liquid and transfer it to a fresh tube that contains a mixture of 5 μL 7.5 M NH_4oAC and 4 μL Glycogen (10 mg/mL). Add 116 μL of 100% EtOH, mix by vortexing and place the tubes at -80 °C for 30 minutes or -20 °C overnight to precipitate the DNA.

[8] While the DNA is precipitating, cast a 12% Urea sequencing gel (19.2 g Urea, 4 mL 5x TBE, 12 mL 40% Acrylamide up to 40 mL in dH_20). Microwave mixture for exactly 8 seconds and shake until all the urea has gone into solution. Add 200 μL 10% APS and 40 μL TEMED, mix well and then use capillary action to cast the gel, tapping gently on the glass plates to avoid creating bubbles. Once solidified, set up the gel in the DNA sequencing electrophoresis apparatus and add 1x TBE as the running buffer. Start the gel pre-heating when you are ready to process your samples.

[9] Take the samples from the -80 °C freezer and clarify (13,000 RPM, 20 minutes, 4 °C). Pipette of the supernatant into the radioactive trash, then dry the samples in a speed vacuum for 10 minutes.

[10] Add ~5-9 μL of Formamide + NaOH buffer, vortex for 5 minutes, then boil at 95 °C for 3 minutes in a heated sand block. Immediately place the samples on ice, then load the pre-heated gel. Once the samples have run the desired length, dry the gel and expose.

9. Est1/Est2 Co-Immunoprecipitation:

[1] Start by generating Est2-ProA (Friedman and Cech, 1999) using the T'n'T quick coupled Transcription & Translation Protocol (Promega)

[2] Incubate at 30 °C for 90 minutes, then put on ice.

[3] While performing T'n'T rxn, purify WT Est1 (Est1 Purification protocol) and determine its concentration.

[4] Pre-wash ~40 μL of IgG Sepharose 6 Fast Flow Bead slurry 2x with 1mL cold TMG Buffer (3,000 RPM, 30 seconds, 4 °C)

[5] Add 10 μL of Bead Slurry to the 50 μL T'n'T rxn, then nutate overnight at 4 °C in a 1.5mL tube.

[6] Clarify (3,000 RPM, 4 °C, 1 minute), pipette off the T'n'T liquid.

[7] Immediately do 4x washes in 1 mL cold TMG Buffer, clarify as above

(Beads should go from red to completely white in color). After the last wash, pipette off all the liquid, leaving only the beads.

[8] Add Est1 (1-2 μg) to the beads and nutate for 10 minutes at 4 °C

(as a control add an equivalent amount of Est1 to IgG Sepharose that underwent the same wash procedure but was not incubated with the Est2 T'n'T Rxn). Clarify as above, dump supernatant.

[9] Wash beads 3x in 200 μL cold TMG Buffer, clarify as above.

[10] After the final wash, pipette off all the liquid leaving only the beads, add 15 μL 2xSB, boil for 3 minutes at 95 °C, then load onto a 12% SDS-PAGE gel (240 V for 40 minutes).

[11] Transfer to nitrocellulose via Western Blot

(primary antibody = Anti-His secondary antibody = Anti-mouse).

10. Telomere Southern:

[1] Yeast is grown at 30 °C (depending on the strain) to an $O.D._{595}$ ~ 1.0. Clarify the cells (3,000 RPM, 4 °C, 7 minutes), dump the supernatant and resuspend the pellet in 1 mL dH_20.

[2] Transfer the cells to a fresh 1.5 mL tube on ice and re-clarify (2,500 RPM, 2 minutes, 4 °C), remove all the supernatant.

NOTE[15]: The pellet can be stored at -20 °C at this point until you are ready to use them.

[3] Isolate the yeast genomic DNA using the Yeastar kit (Zymo Research).

[4] Perform a 60 μL (final volume) XhoI restriction enzyme digest (enzyme depends on the assay) using 5-10 μg Yeast genomic DNA, 6 μL Buffer #2 (New England Biological), 0.6 μL BSA (10 mg/mL), 1 μL XhoI, up to 60 μL in dH_20. Incubate at 37 °C overnight (or at least 6 hours if in a rush).

[5] While the reaction is incubating, cast a 1% agarose gel by combining 500 mL 1x TAE with 5 g agarose, microwave 5 minutes, cool for 2 minutes and then pour into the gel cast at 4 °C (no EtBr is added at this point).

[6] Make 3 L of 1x TAE (It takes 1.5 L to fill the gel running chamber). Save the last 1.5 L of 1x TAE to replace the buffer later in the experiment.

[7] After the XhoI digest, add 1-5 μL of DNA loading dye directly to the sample and immediately load it onto the 1% agarose gel, flanking each side with a 1 Kb DNA ladder.

[8] Run the gel until the dark blue dye front is ~ 1 inch from the bottom of the gel.

NOTE[16]: If you are running the gel at 120 V, the buffer needs to be changed after 6 hours. If the gels runs at 50 V, the buffer can be changed after 12 hours, at which point the voltage can be increased to 120 V to speed up the process. When changing the buffer, add 80 μL of EtBr to the fresh 1.5 L 1x TAE.

[9] Image the gel to make sure the DNA has migrated to the point you desire.

[10] Denature the DNA in 1 L of Denaturing Buffer, nutating for ~30 minutes or until the DNA disappears when re-imaging the gel.

NOTE[17]: The Denaturing Buffer can be reused up to 5x before a fresh solution should be made.

[11] Neutralize the gel by nutating it in Neutralization Buffer for 30 minutes.

[12] While the gel neutralizes, place a pre-cut nitrocellulose membrane (22.5 cm x 15 cm) in a dish containing dH_20 for 8 seconds, then transfer to a dish containing 20x SSC along with 3 pre-cut pieces of Whatman paper.

[13] When the gel is neutralized, brake off the top (above the wells) and discard, flip the gel over and place it back-side-up on the transfer apparatus. Place the pre-soaked membrane on the gel (label a corner for orientation), removing any bubbles by rolling a test tube over the membrane. Place the 3 pre-soaked pieces of Whatman paper (one-by-one) on top of the membrane, removing the bubbles as above.

[14] Place two equal stacks of brown paper towels (~6 inches high) on top of the Whatman paper and place a small weight (300 g) on the top of the towels.

[15] Allow the transfer to go overnight (or at least 6 hours).

[16] While the samples are transferring make the probes:

1. "Telo" Probe: 5 μL 10 μM "Telo" Probe stock, 5 μL PNK Buffer, 2.5 μL [γ-^{32}P]ATP, 36.5 μL dH_20, 1 μL PNK enzyme. Incubate at 37 °C for 30 minutes, then clean the probe on a mini spin column (5,000 RPM, 5 minutes) and store at -20 °C or use immediately.

2. "Cech Chromo IV" Probe (Control) (Friedman and Cech, 1999): 5 μL 10x Standard buffer, 5 μL W303g DNA template, 5 μL 10 μM "Chromo IV" probe primer, 10 μL dNTPs (dACT = 200 μM final), 1 μL cold dGTP (20 μM final), 5 μL [α-^{32}P]GTP, 18 μL dH_20, 0,5 μL Taq Polymerase, setting up the components in order on ice. Pre-heat the top

of the PCR machine (hot top), perform 62 °C for 35 cycle program and store at -20 °C or use immediately.

[17] After the transfer, take the membrane and soak it in 6x SSC for 15 seconds, then place the membrane on a paper towel to air dry for 2 hours at room temperature.

[18] Crosslink the front and back of the membrane using the “optimal setting” on the Crosslinker machine (Spectro-Linker).

[19] The membrane needs to be pre-hybridized in 25 mL of EKONO Buffer, rotating in a hybridization oven for 1 hour at 60 °C before the addition of the probes.

NOTE[17]: Make sure to roll the membrane so that the front of the membrane is on the inside.

[20] Add the two probes to 25 mL of fresh EKONO buffer, discard the used EKONO buffer before adding the new buffer to the membrane. Allow the membrane to rotate at least 6 hours (preferably overnight) at 60 °C.

[21] Following hybridization the blot needs to be washed after dumping the EKONO buffer in the radioactive waste. Put the membrane in a Pyrex dish for the washes:

1.) Wash #1 = 2xSSC + 0.5% SDS for 5 minutes, dump
2.) Wash #2 = 2xSSC + 0.1% SDS for 15 minutes, dump
3.) Wash #3 = 0.1xSSC + 0.1% SDS for 30 minutes, dump

[22] Rinse the membrane quickly with 0.1xSSC, dump. Cover the membrane in cling wrap and expose.

11. Exo-T or Mung Bean Nuclease Assay:

[1] PNK end-label a 15-base overhang template primer (Make the hybrid template as detailed in the “DNA-Binding Assay” protocol)

[2] Pre-incubate the proteins of choice with the DNA template at room temperature for 5-10 minutes.

[3] Make a Master Mix (10 μL Final volume): 12.5nM hybrid template DNA (1μM stock), 20 ng/μL dIdC, 0.2 μg/μL BSA, 1μL Buffer #4 (New England Biological), 1.25μL 80% Glycerol, up to 10 μL in dH_20.

[4] Add the protein(s) to the Master Mix and DNA (final volume 10μL), incubate at room temperature for 5 minutes.

[5] Add 0.5 μL of ExoT or Mung Bean and incubate an additional 10 minutes at room temperature.

[6] Add 10 μL Formamide NaOH buffer and boil for 3 minutes at 95 °C (quenches reaction).

[7] Put the samples on ice immediately, then load onto a pre-warmed 12% Urea gel (recipe for the urea gel is in “Telomerase extension assay” protocol). Dry the gel and expose.

NOTE[18]: For an ExoT Time-Course:

Test 1hr, 30min, 15min, 10min, 5min, 1min and 0 minutes

Set up 7 reactions, but only add ExoT to the 1hr reaction

After 30 minutes add ExoT to the 30 minute reaction

After 45 minutes add ExoT to the 15 minute reaction

After 50 minutes add ExoT to the 10 minute reaction

After 55 minutes add ExoT to the 5 minute reaction

After 59 minutes add ExoT to the 1 minute reaction

After 1hr add ExoT to the 0 minute reaction

Immediately add Formamide NaOH buffer to all the reactions, boil and load on Urea gel as above.

12. Generating mutant Tlc1 yeast extracts:

[1] Yeast (TCy43) expressing wild-type TLC1 from the pTLC1-LYS2-CEN plasmid were transformed with vectors for bulge-delete, stem-disrupt or stem-compensatory TLC1 RNAs. (pAS557 = Bulge Substitution, pAS558 = Bulge Deletion, pAS561 = Stem Compensatory, pAS562 = Stem Disruption) (Seto *et al*., 2002).

[2] The transformants were plated on simple media plus dextrose plates lacking tryptophan (Trp) and lysine (Lys).

NOTE[19]: Plating on this media maintains the wildtype plasmid, because the cells would die otherwise since they initially need the wildtype Tlc1 in order to grow.

[3] The transformants will take ~2 days to grow at 30 °C (Catch the colonies as early as possible).

[4] Replica plate onto α-Aminoadipate plates to drop out the wildtype colonies that use the Lys2 or Lys5 pathway. Grow at 30 °C.

NOTE[20]: Wildtype cells will always use the Lys2 or Lys5 enzyme pathways because they are more efficient, thus the cells will incorporate the α-Aminoadipate into the Ly2/Ly5 pathway which is lethal.

[5] As a test, replica plate the α-Aminoadipate plates onto Simple media and dextrose plates lacking 1.) Tryptophan 2.) Tryptophan and Lysine to observe if the cells lost the wildtype plasmid. Grow at 30 °C. The cells that grow on plates lacking only tryptophan have lost the wildtype plasmid.

[6] Use the colonies on plates lacking Tryptophan to seed a 50 mL Simple media plus dextrose (SD) culture (Add in 5 mL 10x Amino Acid drop out mix lacking tryptophan). Shake the culture at 30 °C until the $O.D._{595}$ is between 1.5-2.0 (exponential growth before stationary/lag phase).

[7] Add the entire 50 mL culture to 1 L SD (100 mL Amino Acid drop out mix lacking Tryptophan).

[8] Allow the culture to shake at 30 °C until the $O.D._{595}$ is between 1.5-2.0 (Save a 5-10 mL aliquot at this stage to use in a Southern Blot).

[9] Use this culture to seed eight 1 L SD (100 mL Amino Acid drop out mix lacking Tryptophan) flasks to an $O.D._{595}$ of 0.25.

[10] Allow the culture to shake at 30 °C until the $O.D._{595}$ is 1.0 (Save a 5-10 mL aliquot at this stage to use in a Southern Blot).

[11] Clarify the cells (5,000 RPM, 7 minutes, 4 °C) and resuspend in 15 mL of chilled Lysis Buffer. Transfer to a fresh 50 mL conical, clarify (2,500 RPM, 5 minutes, 4 °C), dump the supernatant and snap freeze.

[12] Use the yeast pellet to make a telomerase extract as described in the “Telomerase extract preparation” protocol.

[13] Take the saved samples and perform a southern blot (using the “Southern Blot” protocol with the “Telo” and “Cech Chromo IV” probes) to observe if the samples give the expected telomere shortening phenotype in vivo.

REFERENCES

Agashe VR, Guha S, Chang HC, Genevaux P, Hayer-Hartl M, Stemp M. *et al*. (2004) Function of trigger factor and DnaK in multidomain protein folding: increase in yield at the expense of folding speed. *Cell*, **117**:199–209.

Antonsson C, Whitelaw ML, McGuire J, Gustafsson JA and Poellinger L. (1995) Distinct roles of the molecular chaperone hsp90 in modulating dioxin receptor function via the basic helix-loop-helix and PAS domains. *Mol. Cell. Biol*., **15**:756-65.

Arneric M and Lingner J. (2007) Tel1 kinase and subtelomere-bound Tbf1 mediate preferential elongation of short telomeres by telomerase in yeast. *EMBO J*., **8**:1080-5.

Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, Coker C, Krauskopf A. Kupiec M and McEachern MJ. (2004) A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. *Proc. Nat.l Acad. Sci. U.S.A.,* **101**:8658-63.

Bailly A, Savouret JF, Sallas N and Milgrom E. (1978) Factors modifying the equilibrium between activated and non-activated forms of steroid-receptor complexes. *Eur. J. Biochem*., **88**:623-32.

Baneyx F and Mujacic M. (2004) Recombinant protein folding and misfolding in Escherichia coli. *Nat. Biotechnol*., **22**:1399–1408.

Beckmann RP, Mizzen LE and Welch WJ. (1990) Interaction of HSP70 with newly synthesized proteins: implications for protein folding and assembly. *Science,* **248**:850-4.

Bianchi A, Negrini S and Shore D. (2004) Delivery of yeast telomerase to a DNA break depends on the recruitment functions of Cdc13 and Est1. *Mol. Cell*, **16**:139-46.

Blackburn EH, Greider CW, Henderson E, Lee MS, Shampay J and Shippen-Lentz D. (1989) Recognition and elongation of telomeres by telomerase. *Genome*, **31**:553-60.

Blackburn EH. (1991) Telomeres. *Trends Biochem. Sci*., **16**:378-81.

Borkovich KA, Farrelly FW, Finkelstein DB, Taulien J and Lindquist S. (1989) Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell. Biol.*, **9**:3919-30.

Britt-Compton B, Capper R, Rowson J and Baird DM. (2009) Short telomeres are preferentially elongated by telomerase in human cells. *FEBS Lett*., **583**:3076-80.

Bubulya PA and Spector DL. (2004) "On the move"ments of nuclear components in living cells. *Exp. Cell Res.,* **296**:4-11.

Bukau B and Walker GC. (1989) Cellular defects caused by deletion of the Escherichia coli dnaK gene indicate roles for heat shock protein in normal metabolism. *J. Bacteriol*., **171**:2337-46.

Cech TR. (2004) Beginning to understand the end of the chromosome. *Cell*, **116**:273-9.

Chan A, Boule JB and Zakian VA. (2008) Two pathways recruit telomerase to Saccharomyces cerevisiae telomeres. *PLoS Genet*., 4:e1000236.

Chandra A, Hughes TR, Nugent CI and Lundblad V. (2001) Cdc13 both positively and negatively regulates telomere replication. *Genes Dev*., **15**:404-14.

Chang HC, Kaiser CM, Hartl FU and Barral JM. (2005) De novo folding of GFP fusion proteins: high efficiency in eukaryotes but not in bacteria. *J. Mol. Biol*., **353**:397–409.

Cohn M and Blackburn EH. (1995) Telomerase in yeast. *Science*, **269**:396-400.

Counter CM, Meyerson M, Eaton EN and Weinberg RA. (1997) The catalytic subunit of yeast telomerase. *Proc. Natl. Acad. Sci. USA.*, **94**:9202-7.

Deuerling E, Schulze-Specking A, Tomoyasu T, Mogk A and Bukau B. (1999) Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature,* **400**:693-6.

DeZwaan DC and Freeman BC. (2008) Hsp90: the Rosetta stone for cellular protein dynamics? *Cell Cycle*, **7**:1006-12.

DeZwaan DC and Freeman BC. (2009) The conserved Est1 protein stimulates telomerase DNA extension activity. *Proc. Natl. Acad. Sci. USA.*, **106**:17337-42.

DeZwaan DC, Toogun OA, Echtenkamp FJ and Freeman BC. (2009) The Hsp82 molecular chaperone promotes a switch between unextendable and extendable telomere states. *Nat. Struct. Mol. Biol.*, **16**:711-6.

DeZwaan DC and Freeman BC. (2010) HSP90 manages the ends. *Trends Biochem. Sci.*, **35**: 384-91.

DeZwaan DC and Freeman BC. (2010) Is there a telomere-bound 'EST' telomerase holoenzyme? *Cell Cycle*, **9**:1913-17.

Ellis RJ and van der Vies SM. (1991) Molecular Chaperones. *Annu. Rev. Biochem.*, **60**:321-47.

Ellis RJ. (2001) Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr. Opin. Struct. Biol.*, **11**:114-9.

Ellis RJ. (2007) Protein misassembly: macromolecular crowding and molecular chaperones. *Adv. Exp. Med. Biol.*, **594**:1-13.

Evans SK and Lundblad V. (1999) Est1 and Cdc13 as comediators of telomerase access. *Science*, **286**, 117-20.

Evans SK and Lundblad V. (2002) The Est1 subunit of Saccharomyces cerevisiae telomerase makes multiple contributions to telomere length maintenance. *Genetics,* **162**:1101-15.

Fisher TS, Taggart AK and Zakian VA. (2004) Cell cycle-dependent regulation of yeast telomerase by Ku. *Nat. Struct. Mol. Biol.*, **11**:1198-205.

Flom G, Weekes J and Johnson JL. (2005) Novel interaction of the Hsp90 chaperone machine with Ssl2, an essential DNA helicase in Saccharomyces cerevisiae. *Curr. Genet.*, **47**:368-80.

Forsythe HL, Jarvis JL, Turner JW, Elmore LW and Holt SE. (2001) Stable association of HSP90 and p23, but Not HSP70, with active human telomerase. *J. Biol. Chem.*, **276**:15571-4.

Freeman BC and Yamamoto KR. (2001) Continuous recycling: a mechanism for modulatory signal transduction. *Trends Biochem. Sci.*, **26**:285-90.

Freeman BC and Yamamoto KR. (2002) Disassembly of transcriptional regulatory complexes by molecular chaperones. *Science,* **296**:2232-5.

Friedman, KL and Cech TR. (1999) Essential functions of amino-terminal domains in the yeast telomerase catalytic subunit revealed by selection for viable mutants. *Genes Dev.*, **13**:2863-74.

Friedman KL, Heit JJ, Long DM and Cech TR.(2003) N-terminal domain of yeast telomerase reverse transcriptase: recruitment of Est3 to the telomerase complex. *Mol. Biol. Cell*, **14**:1-13.

Garel JR, Martel A, Muller K, Ikai A, Morishima N and Sutoh K. (1984) Role of subunit interactions in the self-assembly of oligomeric proteins. *Adv. Biophys.*, **18**:91-113.

Gautschi M, Mun A, Ross S and Rospert S. (2002) A functional chaperone triad on the yeast ribosome. *Proc. Natl Acad. Sci. USA.*, **99**:4209–14.

Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Mihon AM, Cruciat CM, *et al*. (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature*, **415**:141-7.

Ghaemmaghami S, Huh WK, Bower K, Howson RW, BelleA, Dephoure N, O'Shea EK, Weissman JS. (2003) Global analysis of protein expression in yeast. *Nature,* **425**:737-41.

Gilson E and Géli V. (2007) How telomeres are replicated. *Nat. Rev. Mol. Cell Biol.* **8**:825-38.

Grandin N, Damon C and Charbonneau M. (2000) Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. *Mol. Cell Biol.*, **20**:8397-408.

Grandin N and Charbonneau M. (2001) HSP90 levels affect telomere length in yeast. *Mol. Genet. Genomics,* **265**:126-34.

Greider CW, Blackburn EH. The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell 1987, 51:887-98.

Hager GL, McNally JG and Misteli T. (2009) Transcription Dynamics. *Mol. Cell,* **35**:741-53.

Hartwell LH, Mortimer RK, Culotti J and Culotti M. (1973) Genetic control of the cell division cycle in yeast: V. Genetic analysis of cdc mutants. *Genetics,* **74**:267-86.

Hemann MT, Strong MA, Hao LY and Greider CW. (2001) The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell,* **107**:67-77.

Hendrick JP and Hartl FU. (1993) Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem*., **62**:349-84.

Holt SE, Aisner DL, Baur J, Tesmer VM, Dy M, Ouellette M, Trager JB, Morin GB, Toft DO, Shay JW, Wright WE and White MA. (1999) Functional requirement of p23 and HSP90 in telomerase complexes. *Genes Dev.*, **13**:817-26.

Hu J, Toft DO and Seeger C. (1997) Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J.*, **16**:59-68.

Hu J, Flores D, Toft D, Wang X and Nguyen D. (2004.) Requirement of heat shock protein 90 for human hepatitis B virus reverse transcriptase function. *J. Virol.*, **78**:13122-31.

Hughes TR, Evans SK, Wellbaecher RG and Lundblad V. (2000) The Est3 protein is a subunit of yeast telomerase. *Curr. Biol*., **10**:809-12.

Hughes TR, Weilbaecher RG, Walterscheid M and Lundblad V. (2000) Identification of the single-strand telomeric DNA binding domain of the Saccharomyces cerevisiae Cdc13 protein. **Proc. Natl Acad. Sci. USA**., **97**:6457–62.

Hur E, Kim HH, Choi SM, Kim JH, Yim S, Kwon HJ, Choi Y, Kim DK, Lee MO and Park H. (2002) Reduction of hypoxia-induced transcription through the repression of hypoxia-inducible factor-1alpha/aryl hydrocarbon receptor nuclear translocator DNA binding by the 90-kDa heat-shock protein inhibitor radicicol. *Mol. Pharmacol*., **62**:975-82.

Johnson JL, Halas A and Flom G. (2007) Nucleotide-dependent interaction of Saccharomyces cerevisiae Hsp90 with the cochaperone proteins Sti1, Cpr6, and Sba1. *Mol. Cell. Biol*., **27**:768-76.

Johnson JL and Brown C. (2009) Plasticity of the HSP90 chaperone machine in divergent eukaryotic organisms. *Cell Stress Chaperones*, **14**:83-94.

Kaiser CM, Chang HC, Agashe VR, Lakshmipathy SK, Etchells SA, Hayer-Hartl M. *et al*. (2006). Real-time observation of trigger factor function on translating ribosomes. *Nature*, **444**:455–60.

Kelleher C, Kurth I and Lingner J. (2005) Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. *Mol. Cell Biol.*, **25**:808-18.

Keppler BR, Grady AT and Jarstfer MB. (2006) The biochemical role of the heat shock protein 90 chaperone complex in establishing human telomerase activity. *J. Biol. Chem.*, **281**:19840-8.

Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV and Gottesman MM. (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science*, **315**:525–8.

Konieczny I and Zylicz M. (1999) Role of bacterial chaperones in DNA replication. *Genet. Eng.*, **21**:95-111.

Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, Li J, Pu S, Datta N, Tikuisis AP, *et al.* (2006) Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. *Nature*, **440**:637-43.

Kurland CG, Hughes D and Ehrenberg M. (1996) Limitations of translational accuracy. In Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhart, F. C., ed), pp. 979–1004, ASM Press, Washington, DC.

Kushner DJ. (1969) Self-assembly of biological structures. *Bacteriol. Rev.*, **33**:302-45.

Lendvay TS, Morris DK, Sah J, Balasubramanian B and Lundblad V. (1996) Senescence mutants of Saccharomyces cerevisiae with a defect in telo- mere replication identify three additional EST genes. *Genetics*, **144**:1399–1412.

Li S, Makovet S, Matsuguchi T, Blethrow JD, Shokat KM and Blackburn EH. (2009) Cdk1-dependent phosphorylation of Cdc13 coordinates telomere elongation during cell-cycle progression. Cell, **136**:50-61.

Liang ST, Xu YC, Dennis P and Bremer H. (2000) mRNA composition and control of bacterial gene expression. *J. Bacteriol.*, **182**:3037–44.

Linger BR and Price CM. (2009) Conservation of telomere protein complexes: shuffling through evolution. *Crit. Rev. Biochem. Mol. Biol*., **44**:434-46.

Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V and Cech TR. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, **276**:561-7.

Lippincott-Schwartz J, Roberts TH and Hirschberg K. (2000) Secretory protein trafficking and organelle dynamics in living cells. *Annu. Rev. Cell. Dev. Biol.*, **16**:557–89.

Livengood AJ, Zaug AJ and Cech TR. (2002) Essential Regions of Saccharomyces cerevisiae Telomerase RNA: Separate Elements for Est1p and Est2p Interaction. *Mol. Cell Biol*., **22**:2366-74.

Lundblad V and Szostak JW. (1989) A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell*, **57**:633-43.

Lundblad V and Szostak JW. (1993) A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell*, **57**:633-43.
Marcand S, Brevet V and Gilson E. (1999) Progressive cis-inhibition of telomerase upon telomere elongation. *EMBO J.*, **18**:3509-19.
Marcand S, Brevet V, Mann C and Gilson E. (2000) Cell cycle restriction of telomere elongation. *Curr. Biol.*, **10**:487-90.
Mathews MB, Sonenberg N and Hershey JWB. (2000) Origins and principles of translational control. In Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B. & Mathews, M. B., eds), pp. 1–31, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mayer MP and Bukau B. (2005) HSP70 chaperones: cellular functions and molecular mechanism. *Cell Mol. Life Sci.*, **62**:670-84.
McClintock B. (1941) The stability of broken ends of chromosomes in *Zae mays. Genetics*, **26**:234-82.
McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M and Frydman J. (2007) Diverse cellular functions of the HSP90 molecular chaperone uncovered using systems approaches. *Cell*, **131**:121-35.
Meier B, Driller L, Jaklin S and Feldmann HM. (2001) New function of CDC13 in positive telomere length regulation. *Mol. Cell. Biol.*, 21:4233-45.
Millson SH, Truman AW, King V, Prodromou C, Pearl LH and Piper PW. (2005) A two-hybrid screen of the yeast proteome for HSP90 interactors uncovers a novel HSP90 chaperone requirement in the activity of a stress-activated mitogen-activated protein kinase, Slt2p (Mpk1p). *Euk. Cell*, **4**:849-60.
Mitton-Fry RM, Anderson EM, Theobald DL, Glustrom LW and Wuttke DS. (2004) Structural basis for telomeric single-stranded DNA recognition by yeast Cdc13. *J. Mol. Biol.*, **338**:241-55.
Minton AP. (2006) How can biochemical reactions within cells differ from those in test tubes? *J. Cell Sci.*, **119**:2863-9.
Misteli T. (2001) Protein dynamics: implications for nuclear architecture and gene expression. *Science*, **291**:843-47.
Mitchison TJ. (1996) Self-organization of polymer-motor systems in the cytoskeleton. *Philos. Trans. R Soc. Lond. B Biol. Sci.*, **336**:99-106.
Morin GB. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, **59**:521-9.
Mozdy AD and Cech TR. (2006) Low abundance of telomerase in yeast: implications for telomerase haploinsufficiency. *RNA*, **12**:1721-37.
Müller L, Schaupp A, Walerych D, Wegele H and Buchner J. (2004) HSP90 regulates the activity of wild type p53 under physiological and elevated temperatures. *J. Biol. Chem.*, **279**:48846-54.
Nakada D, Matsumoto K and Sugimoto K. (2003) ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism. *Genes Dev.*, **17**:1957-62.
Nathan DF and Lindquist S. (1995) Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. *Mol. Cell. Biol.*, **15**:3917-25.
Netzer WJ and Hartl FU. (1997) Recombination of protein domains facilitated by co-translational folding in eukaryotes. *Nature*, **388**:343–9.

Niu H, Xia J and Lue NF. (2000) Characterization of the interaction between the nuclease and reverse transcriptase activity of the yeast telomerase complex. *Mol. Cell Biol*., **20**:6806-15.

Nugent CI, Hughes TR, Lue NF and Lundblad V. (1996) Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science*, **274**:249-52.

Olovnikov AM. (1973) A theory of marginotomy. *J. Theor. Biol.*, **41**:181-90.

Osterhage JL, Talley JM and Friedman KL. (2006) Proteasome-dependent degradation of Est1 regulates the cell cycle-restricted assembly of telomerase in Saccharomyces cerevisiae. *Nat. Struct. Mol. Biol*., **13**:720-8.

Pavlou AK and Reichert JM. (2004) Recombinant protein therapeutics—success rates, market trends and values to 2010. *Nat. Biotechnol*., **22**:1513–9.

Pedersen S. (1984) Escherichia coli ribosomes translate in vivo with variable rate. *EMBO J*., **3**:2895–8.

Pennock E, Buckley K and Lundblad V. (2001) Cdc13 delivers separate complexes to the telomere for end protection and replication. *Cell*, **104**:387-96.

Perham RN. (1975) Self-assembly of biological macromolecules. *Philos. Trans. R Soc. Lond. B Biol. Sci*., **272**:123-36.

Picard D. (2006) Intracellular dynamics of the Hsp90 co-chaperone p23 is dictated by Hsp90. *Exp. Cell Res.*, **312**:198-204.

Porter SE, Greenwell PW, Ritchie KB and Petes TD. (1996) The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in Saccharomyces cerevisiae. *Nucleic Acids Res*., **24**:582-5.

Powers MV and Workman P. (2006) Targeting of multiple signalling pathways by heat shock protein 90 molecular chaperone inhibitors. *Endocr. Relat. Cancer.*, **13**:S125-135.

Pratt WB. (1993) The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J. Biol. Chem*., **268**:21455-8.

Pratt WB and Toft DO. (2003) Regulation of signaling protein function and trafficking by the HSP90/HSP70-based chaperone machinery. *Exp. Biol. Med*., **228**:111-33.

Prescott J and Blackburn EH. (1997) Functionally interacting telomerase RNAs in the yeast telomerase complex. *Genes Dev.*, **11**:2790-800.

Puglisi A, Bianchi A, Lemmens L, Damay P and Shore D. (2008) Distinct roles for yeast Stn1 in telomere capping and telomerase inhibition. *EMBO J*., **27**:2328-39.

Qi H and Zakian VA. (2000) The Saccharomyces telomere-binding protein Cdc13 interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. *Genes Dev*., **14**:1777-88.

Redon S, Reichenbach P and Lingner J. (2007) Protein RNA and protein protein interactions mediate association of human EST1A/SMG6 with telomerase. *Nucleic Acids Res.*, **35**:7011-22.

Reichenbach P, Hoss M, Azzalin CM, Nabholz M, Bucher P and Lingner JA. (2003) A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr. Biol*., **13**:568–74.

Richter K, Hendershot LM and Freeman BC. (2007) The cellular world according to HSP90. *Nat. Struct. Mol. Biol*., **14**:90-4.

Ruusala T, Andersson D, Ehrenberg M and Kurland CG. (1984) Hyper-accurate ribosomes inhibit growth. *EMBO J.*, **3**:2575–80.
Sanchez ER, Meshinchi S, Schlesinger MJ and Pratt WB. (1987) Demonstration that the 90-kilodalton heat shock protein is bound to the glucocorticoid receptor in its 9S nondeoxynucleic acid binding form. *Mol. Endocrinol.,* **1**:908-12.
Seto AG, Livengood AJ, Tzfati Y, Blackburn EH and Cech TR. (2002) A bulged stem tethers Est1p to telomerase RNA in budding yeast. *Genes Dev.*, **16**:2800-12.
Schramke V, Luciano P, Brevet V, Guillot S, Corda Y, Longhese MP, Gilson E and Géli V. (2004) RPA regulates telomerase action by providing Est1p access to chromosome ends. *Nat. Genet.,* **36**:46-54.
Shampay J, Szostak JW and Blackburn EH. (1984) DNA sequences of telomeres maintained in yeast. *Nature,* **310**:154-7.
Shampay J and Blackburn EH. (1988) Generation of telomere-length heterogeneity in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.,* **85**:534-8.
Shaknovich R, Shue G, Kohtz DS. (1992) Conformational activation of a basic helix-loop-helix protein (MyoD1) by the C-terminal region of murine HSP90 (HSP84). *Mol. Cell Biol.,* **12**:5059-68.
Shay JW and Wright WE. (1996) Telomerase activity in human cancer. *Curr. Opin. Oncol.* **8**:66-71.
Shore D and Bianchi A. (2009) Telomere length regulation: coupling DNA end processing to feedback regulation of telomerase. *EMBO J.,* **28**:2309-22.
Siller E, DeZwaan DC, Anderson JF, Freeman BC and Barral JM. (2010) Slowing bacterial translation speed enhances eukaryotic protein folding efficiency. *J. Mol. Biol.,* **396**:1310-8.
Singer MS and Gottschling DE. (1994) TLC1: template RNA component of Saccharomyces cerevisiae telomerase. *Science*, **266**:404-9.
Smith CD, Smith DL, DeRisi JL and Blackburn EH. (2003) Telomeric protein distributions and remodeling through the cell cycle in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **14**:556-70.
Smogorzewska A and de Lange T. (2004) Regulation of telomerase by telomeric proteins. *Annu. Rev. Biochem.,* **73**:177-208.
Snow BE, Erdmann N, Cruickshank J, Goldman H, Gill RM, Robinson MO and Harrington L. (2003) Conservation of the telomerase protein Est1p in humans. *Curr. Biol.,* **13**:698–704.
Stavreva DA, Muller WG, Hager GL, Smith CL and McNally JG. (2004) Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. *Mol. Cell Biol.,* **24**:2682-97.
Steiner BR, Hidaka K and Futcher B. (1996) Association of the Est1 protein with telomerase activity in yeast. *Proc. Natl. Acad. Sci. USA.,* **93**:2817-21.
Stellwagen AE, Haimberger ZW, Veatch JR and Gottschling DE. (2003) Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. *Genes Dev.*, **17**:2384-95.
Suswam EA, Li YY, Mahtani H and King PH. (2005) Novel DNA-binding properties of the RNA-binding protein TIAR. *Nucleic Acids Res.,* **33**:4507-18.
Taggart AK, Teng SC and Zakian VA. (2002) Est1 as a cell cycle-regulated activator of telomere-bound telomerase. *Science,* **297**:1023-6.

Taggart AK and Zakian VA. (2003) Telomerase: what are the Est proteins doing? *Curr. Opin. Cell Biol*., **15**:275-80.

Teixeira MT, Arneric M, Sperisen P and Lingner J. (2004) Telomere length homeostasis is achieved via a switch between telomerase-extendible and –non extendable states. *Cell*, **117**:323-35.

Toogun OA, Zeiger W and Freeman BC. (2007) The p23 molecular chaperone promotes functional telomerase complexes through DNA dissociation. *Proc. Natl. Acad. Sci. U.S.A.*, **104**:5765-70.

Toogun OA, DeZwaan DC and Freeman BC. (2008) The HSP90 molecular chaperone modulates multiple telomerase activities. *Mol. Cell. Biol*., **28**:457-67.

Trinkle-Mulcahy L and Lamond AI. (2007) Toward a high-resolution view of nuclear dynamics. *Science*, **318**:1402-07.

Tseng SF, Lin JJ and Tseng SC. (2007) The telomerase-recruitment domain of the telomere binding protein Cdc13 is regulated by Mec1p/Tel1p-dependent phosphorylation. *Nucleic Acids Res.,* **34**:6327-36.

Vitra-Pearlman V. Morris DK and Lundblad V. (1996) Est1 has the properties of a single-stranded telomere end-binding protein. *Genes Dev*., **10**:3094-104.

Wang MJ, Lin YC, Pang TL, Lee JM, Chou CC and Lin JJ. (2000) Telomere-binding and Stn1pinteracting activities are required for the essential function of Saccharomyces cerevisiae Cdc13p. *Nucleic Acids Res.*, **28**:4733–41.

Wang F, Podell ER, Zaug AJ, Yang Y, Baciu P, Cech TR and Lei M. (2007) The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature,* **445**:506-10.

Walerych D, Kudia G, Gutkowska M, Wawrzynow B, Muller L, King FW, Helwak A, Boros J, Zylicz A and Zylicz M. (2004) HSP90 chaperones wild-type p53 tumor suppressor protein. *J. Biol. Chem.,* **279**:48836-45.

Watson JD. (1972) Origin of concatemeric T7 DNA. *Nat. New Biol*., **239**:197-201.

Wegele H, Muller L and Buchner J. (2004) HSP70 and HSP90--a relay team for protein folding. *Rev. Physiol. Biochem. Pharmacol.,* **151**:1-44.

Whitesell L and Lindquist SL. (2005) HSP90 and the chaperoning of cancer. *Nat. Rev. Cancer*, **5**:761-72.

Wilhelmsson A, Cuthill S, Denis M, Wikstrom AC, Gustafsson JA and Poellinger L. (1990) The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. *EMBO J.,* **9**:69-76.

Zappulla DC, Goodrich K and Cech TR. (2005) A miniature yeast telomerase RNA functions in vivo and reconstitutes activity in vitro. *Nat. Struct. Mol. Biol.,* **12**:1072-7.

Zengel JM, Young R, Dennis PP and Nomura M. (1977) Role of ribosomal protein S12 in peptide chain elongation: analysis of pleiotropic, streptomycinresistant mutants of Escherichia coli. *J. Bacteriol*., **129**:1320–9.

Zhang G, Hubalewska M and Ignatova Z. (2009) Transient ribosomal attenuation coordinates protein synthesis and co-translational folding. *Nat. Struct. Mol. Biol*., **16**:274–80.

Zhao R, Davey M, Hsu YC, Kaplanek P, Tong A, Parsons AB, Krogan N, Cagney G, Mai D, Greenblatt J, Boone C, Emili A and Houry WA. (2005) Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the HSP90 chaperone. *Cell,* **120**:715-27.

Zhou J, Hidaka K and Futcher B. (2000) The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA. *Mol. Cell Biol*., **20**:1947-55.

Ziemienowicz A, Konieczny I and Hubscher U. (2001) Calf thymus Hsc70 and Hsc40 can substitute for DnaK and DnaJ function in protein renaturation but not in bacteriophage DNA replication. *FEBS Lett*., **507**:11-15.

APPENDIX A*

BIOCHEMICAL CHARACTERIZATION OF FULL LENGTH CDC13 PURIFIED FROM SmP *E. coli*

Misfolding of eukaryotic proteins upon recombinant production in bacteria has placed great limitations on their biochemical and structural analyses and their therapeutic utilization (Baneyx *et al*., 2004; Pavlou *et al*., 2004). In bacteria, folding of polypeptide nascent chains can be delayed relative to their synthesis ("posttranslational" folding), a process that may promote misfolding of certain recombinant proteins (Netzer and Hartl, 1997; Agashe *et al*., 2004). In contrast, the eukaryotic cytosol appears to be highly capable of efficiently folding protein domains as they emerge from the ribosome ("cotranslational" folding) (Netzer and Hartl, 1997; Agashe *et al*., 2004; Chang *et al*., 2005). Kingdom-specific molecular chaperones have been demonstrated to support each of these distinct folding regimes, including trigger factor (TF) in bacteria (Agashe *et al*., 2004; Kaiser *et al*., 2006) and the ribosome-associated complex in fungi (Gautschi *et al*., 2002). In addition to their different chaperone complements, a major difference between bacteria and eukaryotes is their translation speed. In Escherichia coli, polypeptide elongation rates vary from ~10 amino acids per second (aa/s) during slow growth to ~20 aa/s during fast growth (Pedersen, 1984; Liang *et al*., 2000). In contrast, elongation rates in eukaryotes are thought to be fairly constant and considerably slower (3– 8 aa/s)

* Data presented in this chapter were originally published in Siller E, **DeZwaan DC**, Anderson JF, Freeman BC and Barral JM. (2010) Slowing bacterial translation speed enhances eukaryotic protein folding efficiency. *J. Mol. Biol.*, 396:1310-8. I performed all of the presented experiments in this study.

(Matthews *et al.*, 2000). Although ribosomal pausing at rare codons along mRNAs encoding particular proteins has been shown to affect their activities, (Kimchi-Sarfaty *et al.*, 2007; Zhang *et al.*, 2009) the effect of general variations in polypeptide synthesis rates on protein folding efficiency has remained largely unexplored. In this work, I aimed to study the impact of global changes in protein synthesis rates on de novo protein folding by utilizing streptomycin (Sm)-pseudodependent (SmP) ribosomes of *E. coli*, (Zengel *et al.*, 1977) whose polypeptide elongation rates can be modulated by varying the concentration of Sm present in the growth medium.

SmP ribosomes contain mutations in protein S12 of their decoding center (see Materials and Methods) (Kurland *et al.*, 1996). In the absence of Sm, bacteria harboring these ribosomes display a "hyperaccurate" phenotype, with considerable reduction in translation rates (~5 aa/s) and ~20-fold increase in the accuracy of amino acid incorporation compared to wild type (Ruusala *et al.*, 1984). Addition of Sm relieves this phenotype in a concentration-dependent fashion, restoring translation speed to nearly wild-type levels, as reflected by restoration of growth rates (which correlate directly with protein synthesis speed) (Ruusala *et al.*, 1984), albeit with a concomitant ~7-fold increase in misincorporation rates compared to wild type (Ruusala *et al.*, 1984). Utilization of SmP ribosomes allowed us to focus on the effects of decreased polypeptide elongation rates on protein folding, since for every comparison between bacteria harboring slowribosomes (without Sm) and bacteria harboring fast ribosomes (with Sm), all other experimental parameters were identical and constant.

Previous data had established that firefly luciferase synthesized at slower speeds was more capable of acquiring its native state (Siller *et al.*, 2010), I wished to determine

whether this effect was generally applicable to eukaryotic proteins prone to aggregation when synthesized in bacterial systems. I investigated whether this approach could be successfully applied to large multidomain eukaryotic proteins previously shown to be inefficiently folded to their native state upon recombinant production in bacteria. I selected the telomere binding protein Cdc13 from *S. cerevisiae* (105 kDa), a protein essential for telomere maintenance that protects chromosome ends from damage and recruits the telomerase complex (Nugent *et al*., 1996; Pennock *et al*., 2001). Cdc13 contains three distinct regions: an N-terminal telomerase recruitment domain, a central DNA binding domain, and a C-terminal capping region (Wang *et al*., 2000; Chandra *et al*., 2001). The central DNA binding domain of Cdc13 has been expressed as a soluble and active species in E. coli, which has facilitated its biochemical and structural analyses (Mitton-Fry *et al*., 2004). However, these analyses for the full length protein have been hindered by the inability of wild-type bacteria to yield native material. Similar to firefly luciferase and the GFP fusion proteins, it was observed that most of the full-length Cdc13 protein was present in the soluble fraction when synthesized by SmP ribosomes under slow translation conditions (Siller *et al*., 2010). To assess whether the full length Cdc13 produced in this manner was indeed native, I purified it (Figure 20) from the soluble fraction and compared its DNA binding activity to that of the central DNA binding domain (DBD) alone by electromobility shift assay (EMSA) (Toogun *et al*., 2007; Figure 21). I found that the purified full length Cdc13 displayed DNA binding affinity and selectivity (i.e., 11-base length requirement) comparable to the well-characterized properties of the central DNA binding domain (Hughes *et al*., 2000). I then examined if Cdc13 produced in the SmP strain, maintained its ability to stimulate telomerase

extension activity. Cdc13 and two Cdc13 point mutants (Cdc13-1 and Cdc13-2) were titrated into telomerase extension assays and found to stimulate extension to the same levels as previously observed and the mutants were able to stimulate comparable to full length Cdc13 (DeZwaan *et al*., 2009; Figure 22).

In this study, I investigated whether the fast polypeptide elongation rates of the bacterial ribosome could be responsible, at least partially, for the poor capacity of *E. coli* to fold proteins of eukaryotic origin, normally translated at the considerably slower speed of the eukaryotic ribosome. I found that, indeed, decreasing bacterial polypeptide elongation rates to rates similar to those of eukaryotes promotes the folding of Cdc13, which is prone to aggregate even when grown at low temperatures (18 °C). I believe that these findings provide a general strategy for the production of recombinant proteins that does not rely on the individual manipulation of coding sequences or on the introduction of specific accessory factors.

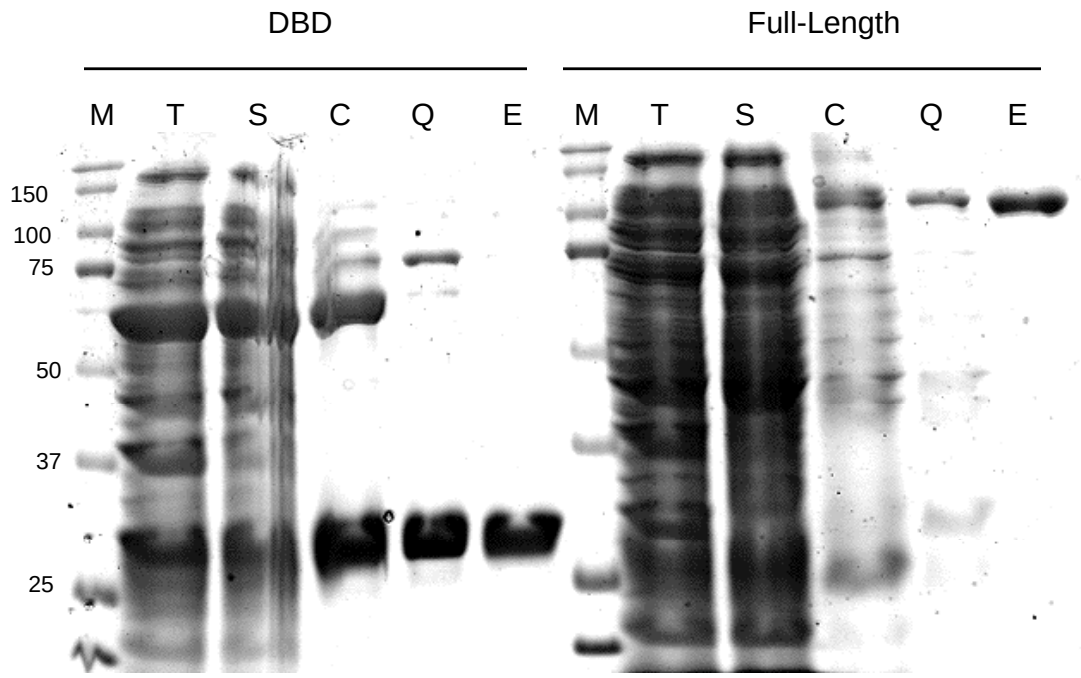

Figure 20. Purification of full-length and the DNA binding domain (DBD) of Cdc13 from streptomycin (Sm)-pseudodependent (SmP) *E. coli*. Total lysates (T) were cleared by centrifugation and the supernatant fraction (S) was subjected to cobalt-affinity (C), MonoQ ion exchange (Q) and Superdex 200 size exclusion (E) resins. Aliquots of each chromatographic step and the final purified proteins (0.5 μg) were co-resolved by SDS-PAGE and the proteins were visualized by staining with Coomassie brilliant blue. Molecular weight markers (M), Cdc13 is ~103 KDa and the DBD is ~30 KDa.

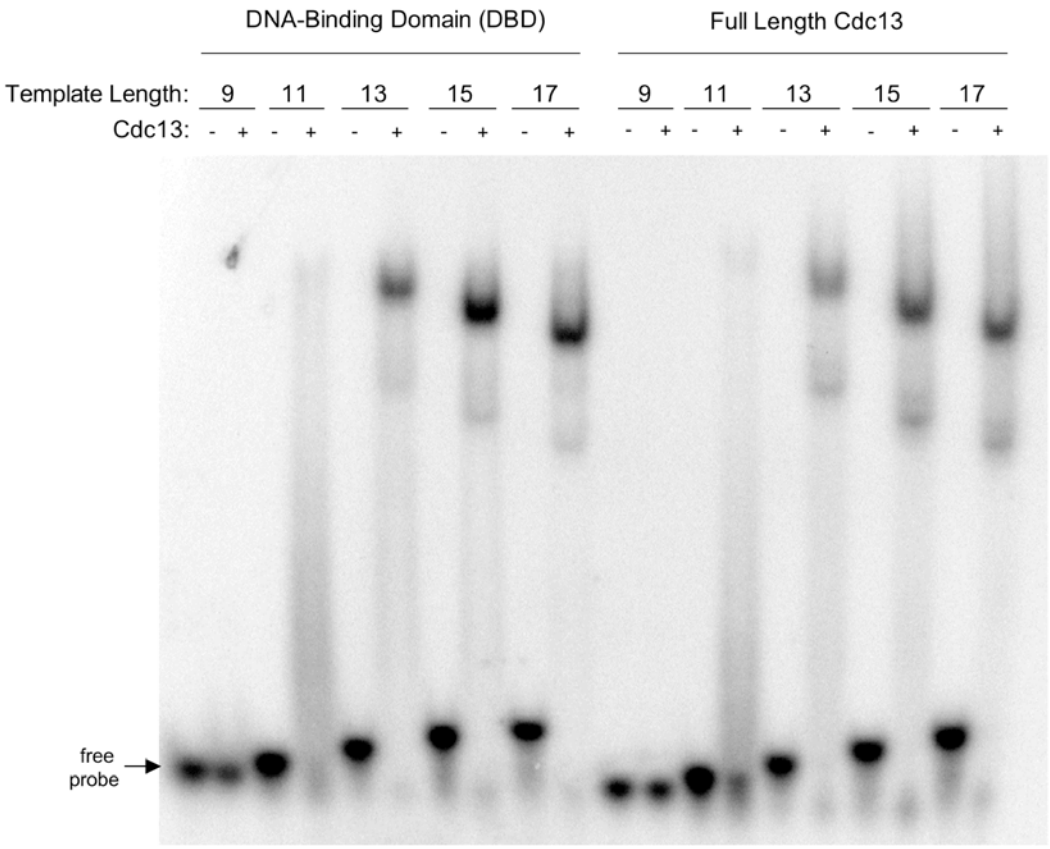

Figure 21. Cdc13 purified from SmP *E. coli* cells binds single-stranded DNA. Electromobility shift assay was used to determine if the purified full-length Cdc13 (F; 100 nM) or its DNA binding domain (D; 100 nM) could bind to single-stranded. The single-stranded DNA length required for binding was determined using telomeric oligonucleotides (50 pM) with the indicated lengths. Arrows mark the approximate position of the free probe.

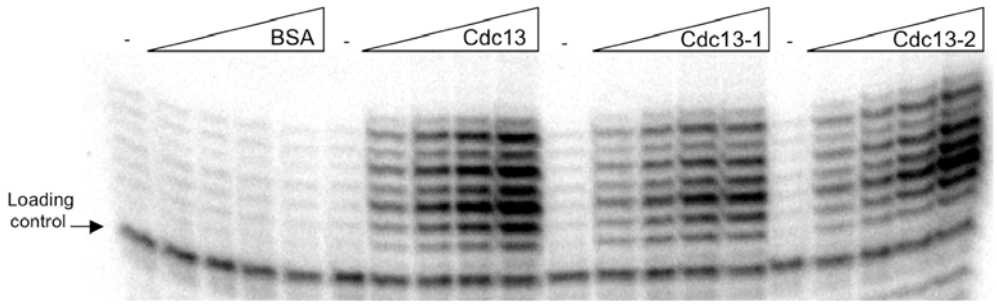

Figure 22. Cdc13 and its various mutants purified from SmP *E. coli* cells stimulate telomerase extension. The Cdc13 effect on telomerase-mediated DNA extension was tested using extracts prepared from YPH499 yeast with an immobilized 7-base 3'-overhang DNA substrate. Telomerase DNA extension reactions were supplemented with BSA, Cdc13, Cdc13-1 or Cdc13-2 (0, 50, 100, 250, 500nM), as marked. To serve as a loading control, an end-labeled 27-base primer was added prior to precipitation of the extension products.

APPENDIX B

BIOCHEMICAL CHARACTERIZATION OF EST3

In *Saccharomyces cerevisae*, the telomerase holoenzyme is composed of three EST (Ever Shorter Telomere) proteins associated with an RNA template called TLC1 (Singer and Gottschling, 1994; Lendvay *et al.*, 1996; Lingner *et al.*, 1997; Hughes *et al.*, 2000). Est3 is one of the essential yeast telomerase co-factors required to promote telomere replication (Lendvay *et al.*, 1996). Previous work has demonstrated that Est3 is recruited to the telomerase complex via the cell cycle regulated Est1 telomerase co-factor. Est3 is also dependent on the presence of Est2 for proper complex association (Hughes *et al.*, 2000; Osterhage *et al.*, 2006). Once the telomerase complex has formed, however, the actual role Est3 plays in telomere regulation is not well understood.

To obtain a better appreciation for the function of Est3 with telomerase, I titrated purified Est3 into telomerase extension reactions that used a 7-base hybrid 3'-overhang template (Figure 23). Est3 was able to stimulate telomerase extension activity, but required higher concentrations than were needed for Cdc13 (Figure 6) or Est1 (Figure 10). Since Est1 and Cdc13 have a positive combinatorial effect on telomerase extension activity, I wondered if the addition of the last EST protein (Est3) would further up-regulate the extension activity observed. Various concentration combinations of the three EST proteins were incubated together, but no additional stimulation was observed (Figure 24). However, since Est3 can up-regulate telomerase on its own (Figure 23), various Est3 point mutants were employed to try to understand its mechanism of action. A triple (ETN) and double (DQ) point mutant that change the bases to alanine were used along

with the K71A mutant which has been shown to give shortened telomeres in vivo, while maintaining its ability to assemble with the telomerase complex (Jennell Talley communication). The K71A and ETN mutants significantly decreased telomerase extension activation, while the DQ mutant up-regulated extension comparable to wildtype (Figure 25). Perhaps the K71A mutation, which displays the greatest telomerase activation defect, disrupts the ability of Est3 to be recruited to telomerase through its interaction with Est1. Additional characterization of all three Est3 mutants will need to be don before we can generate a more complete model to explain the regulatory role of Est3 with telomerase.

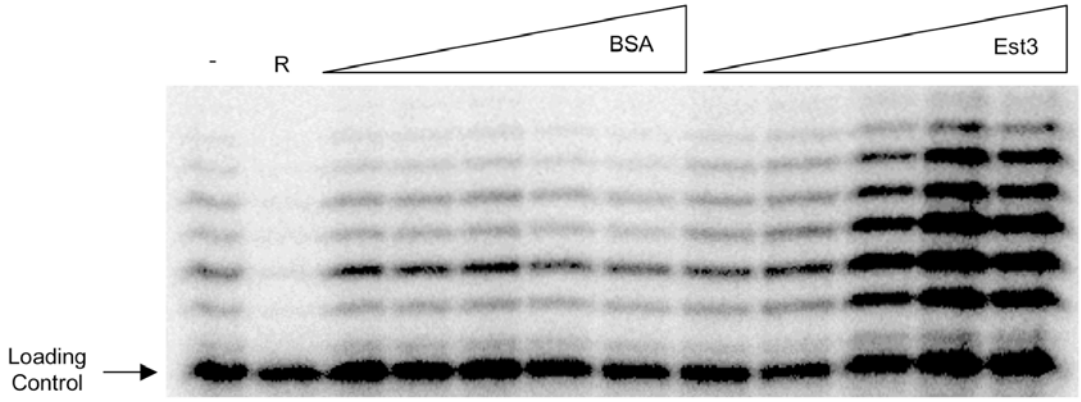

Figure 23. Purified Est3 enhances telomerase DNA extension activity in vitro. The Est3 effect on telomerase-mediated DNA extension was tested using extracts prepared from YPH499 yeast with an immobilized 7-base 3'-overhang DNA substrate. The DNA extension reactions were supplemented with BSA or Est3 (0.5, 1, 2.5, 5, 10 μM), as marked. An RNase control (R) was included to demonstrate that extension was telomerase dependent. To serve as a loading control an end-labeled 27-base primer was added prior to precipitation of the extension products.

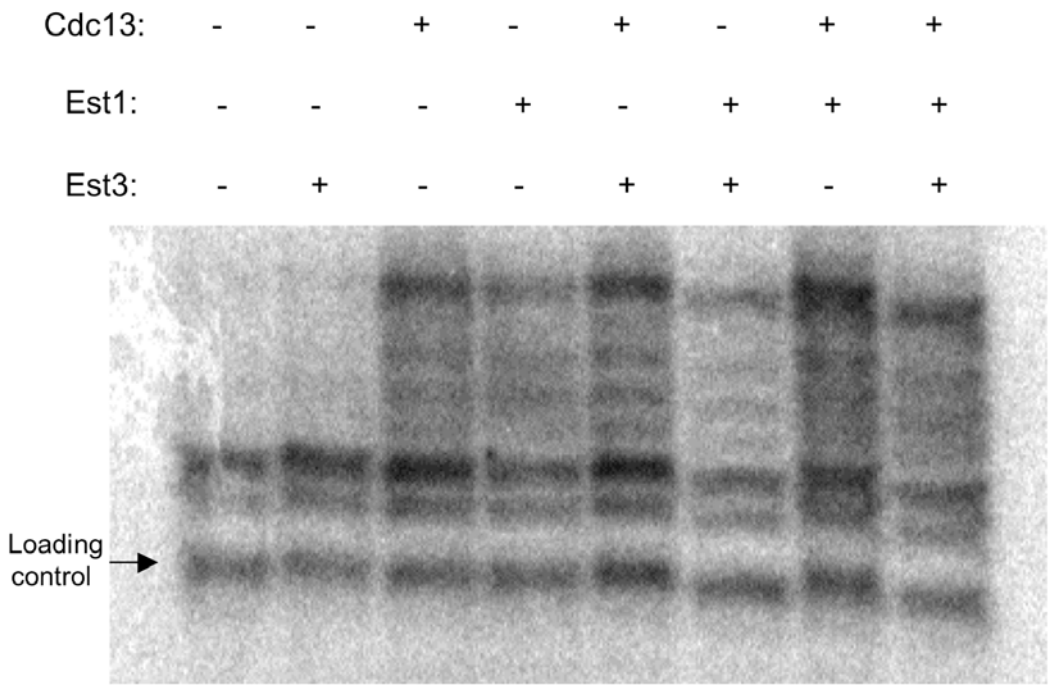

Figure 24. Est3 does not further enhance telomerase stimulation in the presence of Est1 and Cdc13. The effects of the three EST proteins was tested using extracts prepared from YPH499 yeast with an immobilized 7-base 3'-overhang DNA substrate. The reactions were supplemented with 5nM Est1, Est3 and/or Cdc13, as marked. To serve as a loading control an end-labeled 27-base primer was added prior to precipitation of the extension products.

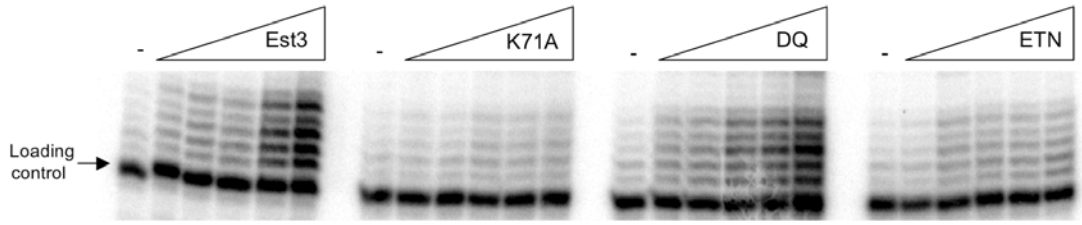

Figure 25. Est3 point mutants adversely affect telomerase extension activity. DNA extension reactions using an immobilized 7-base 3'-overhang DNA substrate were supplemented with either wildtype Est3 or the K71A, DQ or ETN Est3 point mutants (0.25, 0.5, 1, 2.5, 5μM), as marked. To serve as a loading control an end-labeled 27-base primer was added prior to precipitation of the extension products.

AUTHOR'S BIOGRAPHY

Publications

1. DeZwaan, D.C., M.J. Mequio, J.S. Littell, S. Rossbach and V. Pybus (2007) Purification and characterization of enterocin 62-6, a two-peptide bacteriocin produced by a vaginal strain of *Enterococcus faecium*: Potential significance to bacterial vaginosis. **Micro. Eco. Health Dis**., 19, 241-250.
2. Toogun, O.A*., D.C. DeZwaan* and B.C. Freeman (2008) The hsp90 molecular chaperone modulates multiple telomerase activities. **Mol. Cell. Biol**., 28, 457-467.
   *Co-First Authors
3. DeZwaan, D.C. and B.C. Freeman (2008) Hsp90: The Rosetta stone for cellular protein dynamics? **Cell Cycle**, 7, 1-7.
4. DeZwaan, D.C., O.A. Toogun, F.J. Echtenkamp and B.C. Freeman (2009) The Hsp82 molecular chaperone mediates the switch between unextendable and extendable telomere states. **Nat. Struc. Mol. Biol**., 16, 711-716.
5. DeZwaan, D.C. and B.C. Freeman (2009) The conserved Est1 protein stimulates telomerase DNA extension activity. **Proc. Nat'l. Acad. Sci. USA**, 06, 17337-17342.
6. Siller, E., D.C. DeZwaan, J.F. Anderson, B.C. Freeman and J.M. Barral (2010) Slowing bacterial translation speed enhances eukaryotic protein folding efficiency. **J. Mol. Biol**., 396, 1310-1318.
7. DeZwaan, D.C. and B.C. Freeman (2010) Managing the ends by Hsp90. **Trends Biochem. Sci**.. 35, 384-391.
8. DeZwaan, D.C. and B. C. Freeman (2010) Is there an 'EST' telomerase holoenzyme? **Cell Cycle**, 15.
9. Lei, M., J. Sun, Y. Yang, K. Wan, N. Mao, T. Yu, Y. Lin, D. DeZwaan, B.C. Freeman, J.J. Lin and N. Lue. (In Press) Structural bases of dimerization of yeast telomere protein Cdc13 and its interaction with the catalytic subunit of DNA polymerase. **Cell Research.**
10. Talley, J.M., D.C. DeZwaan., B.C. Freeman and K. Friedman (Submitted to **Proc. Nat'l. Acad. Sci.USA**) Est3 Stimulation Of Telomerase Is Dependent Upon Direct Binding To The N-Terminal (TEN) Domain Of Est2.